CORRELATION OF THE HISTOLOGICAL AND CHEMICAL CHANGES IN THE SPLEEN DURING NECROSIS AND AUTOLYSIS.*

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PLATES 62–65.

The original aim of this work was to correlate the chemical changes taking place in tissues undergoing necrosis and autolysis *in vivo,* with the histological picture observed under the same conditions. To do this carefully, it was found desirable to use an organ which was of fairly uniform structure and of uniform chemical composition, and one which could be easily cut off completely from its blood supply without materially affecting the welfare of the animal to which it belonged. For these experiments the spleen was found to be ideal, as it is readily accessible, and its entire blood supply can be easily cut off by ligating its pedicle, after which the organ can be returned to the abdominal cavity in an aseptic condition, to be removed and studied after certain definite intervals.

By using large dogs, moreover, it was hoped that sufficient material could be obtained to make a complete chemical analysis. But the rapidity of the changes *in vivo,* the absorption of the products of tissue disintegration, and the variable pictures produced by numerous complicating and uncontrollable factors, soon showed that it would be necessary to study this organ under conditions in which the autolytic changes were practically free from the influence of such factors as organization, leucocytic infiltration, absorption, inhibition of autolysis by serum, etc.

Aseptic autolysis *in vitro* takes place under simpler conditions, but the histological picture resembles very closely that seen in

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autolysis *in vivo* in the areas farthest removed from the surrounding influences. From the chemical standpoint, also, there was a distinct advantage, for the results *in vitro* could be used to control those obtained *in vivo,* and without some such control, misinterpretation of the results obtained by autolysis *in vivo* would certainly follow.

The most important histological changes that occur during the autolysis that follows cell death are in the nucleus, and since these changes are produced chiefly by specific enzymes, it was thought advisable to study these enzymes and the products which they produce in the dog's spleen in the hope of gaining information which would better explain, at least from a chemical standpoint, some of the histological changes. The details of this part of the work are contained in a previous paper.

Historical.—Up to the present, I have found in the literature no report of a comparative study of the chemical and histological changes in an organ or tissue during autolysis or necrosis. Of the earlier observations on related points, Weigert (1) described the changes undergone by dead tissues, and noted that all cells that had undergone coagulation necrosis had lost their nuclei, but he said that not all cells which had lost their nuclei need necessarily have suffered this change.

Goldmann (2) studied organs kept aseptically in moist chambers at 37° C., and found that the spleen showed no macroscopical changes upon autolysis, but on microscopical examination, after eight days the pulp cells showed changes, while the nuclei in the Malpighian bodies were well preserved. After fourteen days, the trabeculæ were still distinctly differentiated from the pulp, and the nuclei of the Malpighian corpuscles were still retained, but in the remainder of the tissue the nuclei disappeared almost completely.

Klebs (3) observed that in the destruction of the nuclei by mycotic processes two forms of nuclear changes were seen, karyolysis and karyorrhexis. Schmaus and Albrecht (4) found that after ligation of the renal vessels the first change was pyknosis, followed by karyorrhexis, with numerous intermediate variations between this and necrobiosis.

According to Carrière and Vanverts (5) ligation of the pedicle of the spleen results in the death of the organ, which becomes converted into an encapsulated mass of purulent and caseous material with proliferation inward from the capsule. Cultures containing an innocuous colon bacillus, often associated with other harmless organisms, were obtained from the spleen of all the animals experimented upon, both before ligation of the vessels and after the destruction of the spleen was complete. To these organisms the authors attributed the caseous and purulent degeneration that occurred.

Davidsohn (6) transplanted spleens into mice, and later found only pigment rests remaining. Zervos (7) noted that spleens are absorbed in less than a

year after transplantation. Lüdke (8) succeeded in transplanting a rabbit's spleen into the spleens of monkeys, and states that sections showed the included spleens well healed in, but he does not describe the appearances. Carrel (9) in his replantation experiments had accidentally an occlusion of the splenic artery in a dog, and noted that in about a year the spleen had completely disappeared Wells (10) observed that salt solution has a solvent action upon cytoplasm, but that otherwise the autolysis in salt solution proceeded at the same rate as in heated serum, and that splenic tissue after being killed by heat (100° C.) undergoes a very slow change in the body of the rabbit, being attacked only by invading leucocytes, while unheated spleen under the same conditions undergoes rapid autolytic changes with pyknosis, etc.

Although no complete study of the changes occurring in the proteins and nucleoproteins of the spleen during autolysis has been reported, a few observations concerning their constituents have been made. Levene (11) found as end products of autolysis of the spleen, alanine, leucine, aminovalerianic acid, aminobutyric acid, α pyrrolidine-carboxylic acid, phenylalanine, aspartic acid, tyrosine hypoxanthine, xanthine, thymine, and uracyl. Schumm (12) studied autolyzed leukemic spleens and observed a marked drop in the albumose content, and associated with this a threefold increase in non-coagulable nitrogen, which must have come from the coagulable part of the spleen substance as a result of autolysis. He identified as products of autolysis, lysine, leucine, tyrosine, paralactic acid, guanine, xanthine, and hypoxanthine.

Hedin (13) and his coworkers (14) found two distinct proteolytic enzymes (proteases) in the spleen, an α lieno-protease which acts chiefly in alkaline medium, and a β lieno-protease which acts in acid medium. Leathes (15) identified as products of the action of the proteolytic enzyme of the spleen of oxen in acid medium, leucine, tyrosine, aminovalerianic acid, aspartic acid, arginine, histidine, and lysine. The digested fluid also gave the tryptophane reaction, and spectroscopically hematin was found.

Cathcart (16) isolated the products of digestion of coagulated blood serum by α lieno-protease in an alkaline medium, and found histidine, inactive arginine, lysine, tryosine, leucine, alanine, aminovalerianic acid, α pyrrolidine-carboxylic acid, glutamic acid, phenylalanine, and ammonia. Aspartic acid was also probably present, it being formed in large amounts in the digestion of fibrin by the above enzyme. The most marked difference between the action of the two enzymes was in the nature of the arginine found, that obtained by Leathes being optically active.

The changes produced by autolysis in the individual constituents of the spleen have received but slight attention. In putrefaction, lecithin is decomposed into glycerophosphoric acid (17) and choline (18), the latter being further decomposed into methylamine, ammonia, carbonic acid, and methane. Choline is also produced in the autolysis (18) of lecithin, and Coriat believes that brain tissue contains an enzyme capable of producing this change. Meyer (19) found that within three to five weeks the amount of lecithin in pus is decreased by autolysis to about one third.

In autolysis of the liver, pancreas, and thymus the nuclein phosphorus sinks much faster than the lecithin phosphorus, both being converted into the inorganic phosphorus fraction (Morpurgo and Satta (20)).

On account of the difficulty experienced in determining cholesterol quantitatively, only a few observations have been made upon the autolysis of this material. Carbone (21) and Waldvogel (22) believe (what is chemically impossible) that cholesterol originates from lecithin, the latter claiming to have established this by observations upon the digestion of lecithin by sterile liver juice. In experiments which extended over a period of forty-four days he says he obtained a decrease in lecithin and an increase in the fats, fatty acids, and cholesterol, and found a like increase on comparing normal livers with livers showing pathological changes after acute poisoning. Moore (23), however, failed to corroborate these observations in liver autolysis that lasted forty-two days at 37° C. under toluene. In his experiments he obtained 0.038 per cent. of cholesterol before, and 0.0372 per cent. after autolysis. In human splenic pulp he found no essential difference between the cholesterol content in a normal area (0.64 per cent.) and that in an anemic infarcted area (0.58 per cent.).

Practically no chemical study of the iron distribution in the tissues during autolysis has been made. Schmidt (24) found that in anemic infarcts the microchemical reaction for iron was negative or less than that in the surrounding sound tissues. The granular form of iron remaining was distributed over the entire tissue, but was thickest about and in the trabeculæ, which also showed a greater resistance to autolysis or necrosis. A diffuse iron test (Berlin blue and ammonium sulphide reactions) was obtained in the tissues containing granular iron, and Schmidt thinks a solution of iron has taken place, the iron being derived not only from the pigment but also from the entire hemosiderin. Such anemic infarcts often contain hematoidin needles in greater or lesser amounts; and not only is there no iron-containing pigment formed from the blood, but even the preformed iron is removed from the dead tissues. All ordinary embolic splenic infarcts are iron-free before other changes have occurred. Schmidt believes that the iron is not removed by these cells, but that it is destroyed (zerstört) at the site of the infarct. He does not believe that organic combination occurs which might make the microchemical test worthless. His conclusion is that hemosiderin disappears from the tissues on their death, possibly passing through the formation of soluble iron combinations (iron-lecithin), the character of which cannot as yet be stated. It has been suggested that the formation of organic acids in the infarct may explain this disappearance of iron (25).

Methods.—The methods of chemical analysis employed here are identical with those given in a previous paper (26) on the chemical composition of the spleen. Two series of experiments were carried out, those *in vitro* and those *in vivo*.

Before autolysis began in the experiments *in vitro,* samples were taken for the determination of moisture and for the study of the normal histology. Autolysis was carried out under toluene in 0.9 per cent. salt solution at room temperature after the tissue had been cut into small pieces, two to four millimeters square. At the

termination of the autolysis, samples were taken for histological study and the autolytic processes were stopped by means of ten or more volumes of 95 per cent. alcohol in which the material was preserved in the ice chest until the analyses were undertaken.

In the experiments *in vivo,* absolute aseptic technique was used, and the largest dogs obtainable were employed. The pedicle of the spleen was doubly ligated by means of a silk thread, and, after varying intervals had elapsed, the spleen or what remained of it was removed and studied chemically and histologically as in the *in vitro* experiments.

Controls.—As controls on the experiments to be described, the results of chemical analyses and microscopical examinations of normal spleens are here given.

Chemical Composition of Normal Spleen.—Practically all the nuclein nitrogen was either insoluble, precipitable, or coagulable by alcohol. At most the soluble part was but a trace. About 24.1 per cent. of the total phosphorus existed in the form of phosphatids (lecithin, etc.), about 32 per cent. was soluble in water (inorganic phosphates, etc.) and about 40.1 per cent. was insoluble or coagulable (nucleoproteins and other organic coagulable and precipitable combinations). About 5.7 per cent. of the total nitrogen was soluble in water (peptones, amino acids, and ammonia compounds, purins, gelatin, etc.), and 94.3 per cent. insoluble (proteins, nucleoproteins, and other insoluble, precipitable, and coagulable organic nitrogen combinations).

Microscopical Appearance of Normal Spleen.—The trabeculæ were well defined and had nuclei which were distinct and elongated with rounded ends. The nuclei had definitely outlined borders and contained numerous small granules that stained with hematoxylin. The spleen pulp was fairly uniform throughout and had clear oval or round nuclei of various sizes. In nearly all cases these nuclei had a definite outline and contained small hematoxylin granules. In many cases the cytoplasm of individual cells could be distinctly made out, but the granular appearance of the ground substance made this impossible in places. The Malpighian corpuscles were well marked off and distinct and had well defined nuclei which lay close together, were deeply stained by hematoxylin, and contained a definite nucle-

olus. Only rarely could the narrow layer of cytoplasm be made out in these cells. The capsule resembled the trabeculæ in appearance.

The description just given serves for the normal spleens A, B, and C, and for all of the spleens that were subsequently autolyzed *in vitro*.

I. EXPERIMENTS IN VITRO.

Spleen D Autolyzed for Seventeen Hours (Figures 1 and 2).—This spleen was large and before autolysis it had a moist weight of 62.5 gm. and a moisture content of 78.09 per cent. Its dry weight was, therefore, 13.7 gm.

Chemical Analysis of Spleen D after Autolysis for Seventeen Hours.—In the ether-soluble fraction the total material weighed 1.7938 gm., or 13.09 per cent. of the dry weight. The part of this that was insoluble in absolute alcohol weighed 0.0994 gm. and contained 0.0017 gm. of phosphorus (after Neumann oxidation). The cholesterol figure was valueless.

In 1 gm. of dry tissue the lecithin phosphorus weighed 0.00187 gm., which, estimated as lecithin, would amount to 0.0482 gm., and the non-lecithin phosphorus in this fraction weighed 0.00013 gm.

Water-soluble Fraction of Spleen D.—One gm. of dry tissue yielded 0.0103 gm. of nitrogen, of which 0.0021 gm. was precipitated by tannic acid and 0.0082 gm. was not precipitated by tannic acid.

In 1 gm. of dry tissue the water-soluble phosphorus weighed 0.00287 gm.

It was doubtful whether even a trace of purin was present in the water-soluble extractives.

Insoluble Tissue Residue.—One gm. of tissue residue yielded 0.0059 gm. of iron, 0.00689 gm. of sulphur, and 0.00484 gm. of phosphorus. As a result of analyzing about 5 gm. of dry material, 0.00464 gm. of purin nitrogen was found per gram of dry insoluble tissue residue. The total nitrogen in 1 gm. of tissue was 0.1635 gm. In determining this, duplicate analyses, each of 0.25 gm. of tissue, were made.

Microscopical Findings in Spleen D after Autolysis for Seventeen Hours.—After autolysis the trabeculæ and capsules did not differ visibly in appearance from the normal. The splenic pulp, however, showed definite changes. The cells appeared to be fewer in number (few nuclei were present) and stood out with distinct cell boundaries against the reticular framework of the pulp. Their cytoplasm stained well with eosin and contained numerous small vacuoles. The nuclei in most cases were still definitely marked off from the cytoplasm, but stained deeply with hematoxylin and in most cases were homogeneous (pyknosis). A few cells were found in which the nuclei were fragmented, and some nuclei were shaped like crenated red cells. In some cells no nucleus could be seen. The Malpighian corpuscles also showed changes. Cell boundaries were more definite than normally and most of the nuclei stained homogeneously blue. Some nuclei showed fragmentation (karyorrhexis) and some appeared as large rings.

Spleen E Autolyzed for Thirty Hours (Figures 3 and 4).—Before autolysis this spleen had a moist weight of 62.5 gm. and a moisture content of 76.54 per cent. The dry weight was, therefore, 14.67 gm.

Chemical Analysis of Spleen E after Autolysis for Thirty Hours.—In the ether-soluble fraction the total material weighed 2.349 gm., or 16.01 per cent. The part of this that was insoluble in absolute alcohol weighed 0.0280 gm., and contained 0.001 gm. of phosphorus (after Neumann oxidation). The figures obtained for cholesterol were valueless. The lecithin phosphorus found in 1 gm. of dry tissue weighed 0.00228 gm. which, figured as lecithin, amounts to 0.0587 gm. The non-lecithin phosphorus found in this fraction weighed 0.000014 gm.

Water-soluble Fraction.—In 1 gm. of dry tissue the nitrogen precipitated by tannic acid weighed 0.0079 gm., and the nitrogen not precipitated by tannic acid weighed 0.0268 gm. The total nitrogen in the water-soluble fraction was, therefore, 0.0347 gm. The phosphorus in the water-soluble fraction of 1 gm. of dry tissue weighed 0.0054 gm., and the purin nitrogen determined in 60 per cent. of the total water-soluble extractive, amounted to 0.00039 gm. per gram of dry tissue.

Insoluble Tissue Residue.—In 1 gm. of tissue there was 0.0078 gm. of iron, 0.0078 gm. of sulphur, and 0.006 gm. of phosphorus; and in 1 gm. of dry tissue residue there was 0.006 gm. of purin nitrogen. For the last determination a 4 gm. sample was examined. The total nitrogen was 0.1610 gm. per gram of tissue. In estimating this, duplicate analyses were made, each upon 0.25 gm. of tissue residue.

Microscopical Findings in Spleen E after Autolysis for Thirty Hours.—The definition of the trabeculæ and capsule was not as clear as in spleen D, but the nuclei appeared to be more distinct, not differing much from normal, however. The splenic pulp had suffered a further marked change, and appeared granular with numerous small hematoxylin-stained dots on a diffuse bluish tinted field. The nuclei seemed to have undergone disintegration and fragmentation (karyorrhexis), leaving hematoxylin-staining round and oval globules of various sizes and occasionally a distinct nucleus. The cytoplasm of the cells takes a bluish pink tint, and in some cases these cells appeared merely as shadows.

The Malpighian corpuscles stood out distinctly with their nuclei intact, but in shape some of the cells resembled crenated red corpuscles. The cytoplasm of these cells seemed to have increased in amount and in many cases presented a definite border.

Spleen F Autolyzed for Fifty-three Hours (Figures 5 and 6). Chemical Analysis.—Three spleens which before autolysis had a moist weight of 30, 31.5, and 23.5 gm., and a moisture content of 74.25 per cent., 77.93 per cent., and 77.04 per cent. respectively, were united and used. Their total dry weight was 20.07 gm.

Ether-soluble Material.—The dry weight of this fraction was 2.3085 gm. or 11.5 per cent. Of this the part insoluble in absolute alcohol weighed 0.0787 gm., and contained 0.0093 gm. of phosphorus (after Neumann oxidation). The cholesterol figure obtained was valueless. The lecithin phosphorus found in 1 gm. of dry tissue weighed 0.00157 gm., which, figured as lecithin, amounted to 0.0404 gm. The non-lecithin phosphorus found in this fraction weighed 0.00019 gm.

Water-soluble Fraction.—In 1 gm. of the dry tissue the nitrogen precipitated by tannic acid weighed 0.0056 gm., and the nitrogen not precipitated by tannic acid weighed 0.0319 gm. The total nitrogen in the water-soluble fraction was,

therefore, 0.0375 gm. The phosphorus in the water-soluble fraction of 1 gm. of dry tissue weighed 0.0063 gm., and the purin nitrogen determined in 0.7 gm. of the total water-soluble extractives amounted to 0.00098 gm. per gram of dry tissue.

Insoluble Tissue Residue.—One gm. of tissue residue yielded 0.00437 gm. of iron, 0.00656 gm. of sulphur, and 0.00416 gm. of phosphorus. One gm. of dry tissue residue (a 5.5 gm. sample was analyzed) yielded 0.00448 gm. of purin nitrogen. The total nitrogen was 0.1610 gm. per gram of tissue residue, the analyses being made in duplicate, each on 0.25 gm. of tissue residue.

Microscopical Findings in Spleen F after Autolysis for Fifty-three Hours.—The trabeculæ and capsule could still be distinctly differentiated from the spleen pulp, but the nuclei in the trabeculæ were decreased in number and size, solution seeming to have taken place at the borders, so that the nuclei appeared narrow and long. Well formed nuclei, however, could still be seen in some parts of the trabeculæ. Pyknosis was not clearly observed. The pulp had a homogeneous, granular appearance. Many of these granules or droplets stained conspicuously with hematoxylin. Some nuclei were still visible in the pulp, a few still staining slightly, but the majority remained merely as shadows.

The Malpighian corpuscles could no longer be clearly differentiated from the pulp, but in their stead, large numbers of shadows that stained faintly with hematoxylin were seen. These were aggregated especially closely about the blood vessels and trabeculæ. No intact nuclei were found.

Spleen G after Autolysis for Five Days. Chemical Analysis.—Five spleens, which before autolysis had a moist weight of 10 gm., 20 gm., 15.5 gm., 11 gm., and 45.5 gm., and a moisture content of 77.47 per cent., 76.65 per cent., 76.65 per cent., 79.11 per cent., and 77.21 per cent. respectively, were united and used. Their total dry weight was 23.21 gm.

Ether-soluble Fraction.—The total material weighed 2.6644 gm., or 11.47 per cent. Of this the absolute alcohol-insoluble part weighed 0.0654 gm. and contained 0.00178 gm. of phosphorus (after Neumann oxidation). The cholesterol figures obtained were of no value. The lecithin phosphorus in 1 gm. of dry tissue weighed 0.0014 gm., which, figured as lecithin, amounted to 0.03627 gm. The non-lecithin phosphorus in this fraction weighed 0.00009 gm.

Water-soluble Fraction.—In 1 gm. of dry tissue the nitrogen precipitated by tannic acid weighed 0.00505 gm., and the nitrogen not precipitated by tannic acid weighed 0.02998 gm. The total nitrogen in the water-soluble fraction was, therefore, 0.03503 gm. The phosphorus in the water-soluble fraction of 1 gm. of dry tissue weighed 0.0057 gm. The purin nitrogen in this fraction was not determined.

Insoluble Tissue Residue.—One gm. of tissue residue yielded 0.0075 gm. of iron, 0.00894 gm. of sulphur, and 0.0041 gm. of phosphorus. One gm. of dry tissue residue (about 6 gm. were analyzed) yielded 0.00377 gm. of purin nitrogen. The total nitrogen amounted to 0.1622 gm. per gram of tissue residue, the analyses being made in duplicate, each on about 0.25 gm. of tissue residue.

Hausmann Fractions of Proteins in Tissue Residue.—The following figures were obtained from 1 gm. of dry tissue residue. These figures are the mean of duplicate analyses.

Amid nitrogen	= 0.01311 gm. nitrogen.
Humus nitrogen	= 0.00678 gm. nitrogen.
Diamino nitrogen	= 0.03897 gm. nitrogen.
Monamino nitrogen	= 0.1031 gm. nitrogen.
Total nitrogen figured	= 0.16196 gm. nitrogen.

Microscopical Findings in Spleen G after Autolysis for Five Days.—The trabeculæ and capsule were still differentiated, but not as clearly as in F, and a few nuclei were found which stained deeply with hematoxylin, no fragmentation being visible.

The splenic pulp and Malpigian corpuscles were a mass of diffusely stained granular material, with a slightly greater concentration of hematoxylin-stained granules where the Malpighian corpuscles previously existed. In the pulp proper most of the hematoxylin-stained globules have entirely disappeared, leaving a granular ground substance made up in places of what appear to be the shadows of previously existing cells. On close inspection the nuclear boundaries may be seen in many cases, and in some cases the cell boundaries also. Where the Malpighian corpuscles were formerly located there is a greater number of these cell shadows. The endothelial cells on the surface of the capsule seemed to be perfectly preserved.

Spleen H after Autolysis for Six Days (Figures 7 and 8). Chemical Analysis.—Three spleens, two having together a moist weight of 67 gm., and the third a moist weight of 42 gm., with a moisture content of 77.98 per cent. and 77.72 per cent. for the first two, and 77.17 per cent. for the third, were united and used. The total dry weight was 24.51 gm.

Ether-soluble Fraction.—The total material soluble in ether weighed 2.887 gm., or 11.78 per cent. Of this the part insoluble in absolute alcohol weighed 0.0364 gm. and contained 0.00153 gm. of phosphorus (after Neumann oxidation). Cholesterol found in 1 gm. of dry tissue weighed 0.02162 gm. In making this determination one half of the total of ether-soluble material was used for analysis. The lecithin phosphorus in 1 gm. of dry tissue weighed 0.00133 gm., which, figured as lecithin, amounted to 0.0343 gm. The non-lecithin phosphorus in this fraction weighed 0.000066 gm.

Water-soluble Fraction.—In 1 gm. of dry tissue the nitrogen precipitated by tannic acid weighed 0.00476 gm., and the nitrogen not precipitated by tannic acid weighed 0.03680 gm. The total nitrogen in the water-soluble fraction of 1 gm. was, therefore, 0.04156 gm. The phosphorus in the water-soluble fraction of 1 gm. of the dry tissue weighed 0.00691 gm. and the purin nitrogen determined in 0.6 gm. of the total water-soluble extractives amounted to 0.00072 gm.

Insoluble Tissue Residue.—One gm. of tissue residue yielded 0.003285 gm. of phosphorus, and 1 gm. of dry tissue residue (about 5 gm. were used) yielded 0.00315 gm. of purin nitrogen.

Hausmann Fractions of Proteins in Tissue Residue.—The figures obtained from 1 gm. of dry tissue residue follow. These figures are the mean of duplicate analyses.

Amid nitrogen	=	0.01247 gm. of nitrogen.
Humus nitrogen	=	0.00767 gm. of nitrogen.
Diamino nitrogen	=	0.04158 gm. of nitrogen.
Monamino nitrogen	=	0.10660 gm. of nitrogen.
Total nitrogen	=	0.16832 gm. of nitrogen.

Microscopical Findings in Spleen H after Autolysis for Six Days.—The autolysis had gone even a step farther here than in spleen G, so that, with the exception of an occasional nucleus here and there, practically none could be seen even in the trabeculæ. Otherwise the section resembled that of spleen G, the pulp merely showing shadows of cells with no definite stained nuclei. The form of the nuclei, however, was apparently still visible in some cells.

II. EXPERIMENTS IN VIVO.

Spleen I after Autolysis for Two Days. Chemical Analysis.—A single spleen removed from a dog two days after the pedicle had been completely tied off had a moist weight of 121 gm. and a moisture content (determinations made in duplicate) of 83.84 per cent., making a total dry weight of 19.86 gm.

Ether-soluble Fraction.—The total material weighed 2.1629 gm., or 10.88 per cent. of the dry weight. Of this the part insoluble in absolute alcohol weighed 0.2442 gm. and contained 0.0013 gm. of phosphorus (after Neumann oxidation). The cholesterol found in 1 gm. of dry tissue weighed 0.02175 gm. In making this determination one half of the total material soluble in ether was used for analysis. The lecithin phosphorus found in 1 gm. of the dry tissue weighed 0.000066 gm., which, figured as lecithin, amounted to 0.0017 gm. The non-lecithin phosphorus found in this fraction weighed 0.000023 gm.

Water-soluble Fraction.—In one gm. of dry tissue the nitrogen precipitated by tannic acid weighed 0.0009 gm., and the nitrogen not precipitated by tannic acid weighed 0.00649 gm. The total quantity of nitrogen in the water-soluble fraction of 1 gm. was, therefore, 0.00739 gm. The phosphorus in the water-soluble fraction of 1 gm. of dry tissue weighed 0.00085 gm. An uncertain trace of purin was found in this fraction, three fifths of the total extractives being used for analysis.

Insoluble Tissue Residue.—One gm. of dry tissue residue yielded 0.0070 gm. of iron, 0.00785 gm. of sulphur, and 0.00153 gm. of phosphorus. One gm. of dry tissue residue (about 5 gm. samples being used for duplicate analyses, which agreed well) yielded 0.00056 gm. of purin nitrogen.

Hausmann Fractions of Proteins in Tissue Residue.—The mean figures obtained from duplicate analyses of 1 gm. of dry tissue residue are as follows:

Amid nitrogen	=	0.01197 gm. of nitrogen.
Humus nitrogen	=	0.00784 gm. of nitrogen.
Diamino nitrogen	=	0.03605 gm. of nitrogen.
Monamino nitrogen	=	0.10230 gm. of nitrogen.
Total nitrogen figured	=	0.15816 gm. of nitrogen.

Spleen I after Autolysis for One Day.—The spleen was obtained from a 50 kilo dog, in which the pedicle had been ligated two days previously. The spleen

was soft and of a greenish appearance, but its consistence was such that it could still be cut without disintegration.

On microscopical examination the trabeculæ, from which all vestiges of nuclei had disappeared, could still be differentiated from the pulp, but the staining qualities were about the same in both, affinity for hematoxylin having been lost. The surface of the capsule, however, was invaded superficially by leucocytes and by proliferating connective tissue cells, and probably by endothelial cells, while in the deeper part of the capsule these were absent, only shadow-like tissue forms being seen. The splenic pulp was one mass of granular debris with here and there a stray leucocyte. These were seen especially near the capsule. In appearance the pulp resembled that of spleen G, except that the form of the cells seemed to be better preserved and were seen more distinctly. In certain areas in the pulp, pigment-laden cells containing yellowish and brownish granules were seen. These were especially noticeable near the capsule.

Spleen K after Autolysis for Ten Days.—A dog weighing 24.8 kilos was used for this experiment, the spleen pedicle being tied off by about ten double ligatures. The rectal temperature on the day of the operation and during the succeeding days ranged between 101.9° F. and 104° F. No ill effects were noticed except those consequent to the ether anesthesia. On the tenth day the animal was chloroformed and the post-mortem examination revealed an intensely congested omentum which was adherent to the intestines in several places, and in the peritoneal cavity was seen a thick reddish exudate. The spleen was found in an area of intense congestion and adhesions in the left upper posterior part of the peritoneal cavity. It was encapsulated by a fibrous tissue capsule which when opened revealed a central greenish semi-liquid mass weighing about 6.5 gm. and having a moisture content of 80.81 per cent. (average of duplicate determinations). The left kidney appeared slightly congested and there were small petechiæ on the left diaphragmatic wall. No chemical analysis of this small amount of material was attempted.

Microscopical examination revealed a typical necrotic tissue with no nuclei and only the semblance of the previously existing trabeculæ and splenic pulp. No marked leucocytic invasion was noted, except at the periphery of what appeared to be the remnants of the capsule, where a few leucocytes, connective tissue, and endothelial cells were found. The pulp was diffusely granular and in places were seen what appeared to be the shadows of cells with here and there areas that contained masses of large, rod-shaped organisms.

Spleen L after Autolysis for Twenty Days.—As in previous experiments the splenic pedicle was doubly ligated in a dog weighing 25.8 kilos, and the daily rectal temperature in this animal ranged between 101.2° and 103.8° F. Post-mortem examination on the twentieth day revealed a reddish and congested omentum and, in the peritoneal cavity, a small amount of thick reddish exudate which was less in amount than that seen in the ten day experiment. The spleen remnant was found with omental adhesions in a mass of connective tissue. Incision through this capsule revealed a small amount of faintly reddish creamy liquid, which could not be studied chemically. It had, however, a moisture content of 82.72 per cent. (average of duplicate analyses).

Spleen M Autolyzed for Forty Days.—A dog weighing 25.9 kilos was used. The splenic pedicle was ligated as in previous experiments, and the rectal tem-

perature (taken at intervals during the next forty days), ranged between 101.3° and 105.8° F. On the fortieth day the dog appeared to be in perfect health.

After it had been chloroformed, post-mortem examination revealed no exudate in the peritoneal cavity, but there was still a slight congestion of the omentum. The spleen remnant was completely encapsulated and on section only the dense capsule with a small reddish center was found. No chemical examination could be made upon this material, but its moisture content was found to be 82.53 per cent. (the average of duplicate determinations.)

Upon microscopical examination the splenic remnant was found to be made up of typical scar tissue, interspersed with small areas containing pigment-laden cells. The connective tissue was irregularly arranged and was composed of spindle-shaped cells. In some places the cellular elements predominated, in others the intercellular material. The pigment-laden cells were of large size, contained a small nucleus, and in their cytoplasm was seen a large amount of brownish yellow granular pigment. In one section which appeared to contain the former capsule of the spleen there was a more regular arrangement of the various types of connective tissue, and internal to this were found a large number of large phagocytic pigment cells.

The results of the foregoing analyses are summarized in tables II and III.

The results were as follows:

1. There was a gradual but definite decrease in the total amount of the ether-soluble materials. The amount of cholesterol did not change to an appreciable extent, but the lecithin and phosphorus contents decreased proportionately to the stage of autolysis.

2. There was a gradual but definite and proportionate increase in the total water-soluble nitrogen. The main regular increase was in the fraction not precipitated by tannic acid (polypeptids and amino acids). In the purins which had been split out of the nucleins by autolytic enzymes the increase was slight but regular, but in the fraction precipitated by tannic acid there was no appreciable change.

3. There was an increase in the water-soluble phosphorus which progressed with autolysis, the addition being no doubt partly from the phosphorus set free through autolysis, but mainly from disintegration of nucleins.

4. There was a gradual but regular decrease in the phosphorus contained in the insoluble tissue residue. This was due to the nuclear material going into solution. The iron and sulphur content of this fraction did not reveal any consistent change during autolysis.

5. There was a gradual decrease in the purin nitrogen contained in the insoluble tissue residue (nuclein disintegration).

6. There was a slight but gradual decrease in the total nitrogen

TABLE I.

Changes Produced in the Composition of the Spleen during Autolysis.

Fractions.	Divisions of fractions.	Per cent. of one gram of the original moisture-free tissue.								In vivo.
		Normal spleens.			Autolyzed spleens. In vitro.					
		A	B	C	D	E	F	G	H	I
Ether-soluble fraction	Total	15.47	11.66	15.11	13.09	16.01	11.50	11.47	11.78	10.88
	Absolute alcohol-soluble	13.82	9.57	14.05	12.37	15.82	11.11	11.19	11.63	9.65
	Absolute alcohol-insoluble	1.65	2.09	1.06	0.725	0.19	0.39	0.28	0.15	1.23
	Phosphorus content of absolute alcohol-insoluble	0.048	0.042	0.017	0.012	0.007	0.046	0.007	0.006	0.006
	Cholesterol		1.504						2.16	2.17
	Per cent. of ether-soluble as cholesterol		15.07						18.59	22.52
	Lecithin	6.94	Lost	6.21	4.82	5.86	4.03	3.62	3.43	0.17
	Per cent. of lecithin ether-soluble	50.23	Lost	44.23	39.05	37.05	36.36	32.41	29.49	1.76
	Lecithin phosphorus	0.269		0.241	0.187	0.227	0.156	0.141	0.133	0.006
	Non-lecithin phosphorus	0.050	Lost	0.0147	0.0131	0.0014	0.0187	0.009	0.006	0.0023
Water-soluble fraction	Nitrogen not precipitated by tannic acid	0.396	0.392	0.337	0.821	2.678	3.187	2.998	3.68	0.649
	Nitrogen precipitated by tannic acid	0.576	0.411	0.119	0.212	0.792	0.563	0.504	0.476	0.089
	Purin nitrogen	0.00	0.00	0.00	0.00	0.038	0.097	Lost	0.072	Trace
	Per cent. of total purin nitrogen	0.00	0.00	0.00	0.00	9.734	27.41	Lost	28.02	0.00
	Total nitrogen	0.972	0.803	0.456	1.033	3.47	3.75	3.50	4.156	0.738
	Phosphorus	0.518	0.345	0.269	0.287	0.543	0.632	0.570	0.691	0.085
Insoluble tissue residue	Iron	0.265	0.370	0.983	0.468	0.464	0.253	0.481		0.54
	Sulphur	0.533	0.581	0.600	0.547	0.464	0.380	0.574		0.606
	Phosphorus	0.637	0.381	0.393	0.345	0.356	0.241	0.262	0.194	0.118
	Purin nitrogen	0.261	0.238	0.345	0.369	0.357	0.259	0.241	0.186	0.041
	Total nitrogen	10.96	13.34	13.13	13.02	9.63	9.34	10.40	9.57	12.21

TABLE II.

Changes Produced in the Nitrogen and Phosphorus Distribution of the Spleen during Autolysis.

Fraction.	Divisions of fractions.	Per cent. of the total nitrogen or phosphorus of the original tissue.								
		Normal spleens.			Autolyzed spleens. In vitro.					In vivo.
		A	B	C	D	E	F	G	H	I
Ether-soluble fraction	Absolute alcohol-insoluble phosphorus	3.17	4.11	1.91	1.48	0.61	4.22	0.776	0.606	3.07
	Lecithin phosphorus	17.69		25.75	22.12	20.04	14.31	14.24	12.93	3.02
	Non-lecithin phosphorus	3.29		1.57	1.54	0.123	1.70	0.916	0.636	1.073
Water-soluble fraction	Nitrogen not precipitated by tannic acid	3.32	2.77	2.48	5.84	20.44	24.35	21.57	26.83	5.01
	Nitrogen precipitated by tannic acid	4.83	2.91	0.879	1.51	6.04	4.30	3.63	3.46	0.69
	Purin nitrogen	0.00	0.00	0.00	Trace	0.294	0.748		0.527	Trace
	Phosphorus	34.03	33.09	28.76	33.96	47.83	57.71	57.66	67.05	38.86
Insoluble tissue residue	Phosphorus	41.81	36.51	41.99	40.81	31.41	22.03	26.51	18.82	53.98
	Purin nitrogen	2.19	1.68	2.54	2.62	2.72	1.98	1.73	1.35	0.318
	Total nitrogen	91.89	94.36	96.70	92.66	73.53	71.41	74.82	69.69	94.34

TABLE III.

Per Cent. of Total Nitrogen in Extracted Residue.

Spleens.	Normal.	In vitro 5 days.	In vitro 6 days.	In vivo 2 days.
	C	G	H	I
Amid nitrogen	8.60	8.09	7.40	7.57
Humus nitrogen	5.76	4.19	4.55	4.96
Diamino nitrogen	21.87	24.05	24.68	22.78
Monamino nitrogen	63.71	63.65	63.37	64.65
Total per cent.	99.94	99.98	100.00	99.96

contained in the insoluble tissue residue which, divided into the Hausmann fractions, did not reveal any appreciable difference in the nitrogen distribution in the protein of the tissue residue before or after autolysis.

Autolysis in Vivo.—The results were as follows:

1. There was a decrease in the ether-soluble fraction, which was more marked than the decrease after autolysis *in vitro*. In the phosphatid phosphorus there was a great decrease which was not associated with an increase in the water-soluble phosphorus, presumably because of diffusion of the soluble products of autolysis. As far as could be ascertained there was, however, no appreciable change in the cholesterol determinations. This was discussed in an earlier paper on cholesterol determinations (26).

2. In the total water-soluble nitrogen there was no appreciable increase, but there was a doubling of the nitrogen not precipitated by tannic acid, a decrease in the nitrogen precipitated by tannic acid, and an absence of purin nitrogen.

3. In the water-soluble phosphorus there was a marked decrease due to diffusion.

4. The phosphorus contained in the insoluble tissue residue decreased, but the iron and sulphur content of this fraction were normal, behaving as they did after autolysis *in vitro*.

5. A much greater decrease was noted in the insoluble purin nitrogen.

6. In the total nitrogen or in the Hausmann fractions of the insoluble tissue residue after autolysis, there was no appreciable change.

SIGNIFICANCE OF CHEMICAL CHANGES OCCURRING IN AUTOLYSIS, WITH SPECIAL REFERENCE TO THE HISTOLOGICAL CHANGES OCCURRING COINCIDENTALLY.

In drawing conclusions from microscopical examinations of a tissue we can, at most, notice only the superficial changes, and the main ones occur in the nucleus. With the aid of modern chemistry we are, however, enabled to investigate the cell further and to associate what we see histologically with what actually happens to some, at least, of the chemical components of tissues. As methods of biochemical analysis are still far from perfect, we must be satisfied with a study of such simple components as can be accurately estimated chemically.

When the pathologist finds microscopical changes in a tissue, he associates these with what he has previously seen, but he has no knowledge as to how much of the actual tissue constituents has been removed in the process, whether all are still present but in a changed form, or whether the greater part has been removed, leaving only the mere framework or skeleton; in other words, he has no means of knowing what percentage of the protein, nucleoprotein, lecithin, etc., of the original tissue still exists as such when certain microscopical changes are observable.

A few of these questions are answered by the results of this investigation, and will be taken up here by considering the changes in the chemical constituents which are associated with the microscopically visible pathological changes.

In the normal spleen the nucleins are practically all in forms which are insoluble, coagulable, or precipitable. In autolysis *in vitro* they remain thus in the first stages, even when a high grade of pyknosis is visible microscopically. When karyorrhexis and karyolysis are first seen, a small percentage (about 10 per cent.) of the nuclein nitrogen has become soluble in the form of purin bases, but when karyolysis is completed so that no nuclei remain that stain, only about 28 per cent. of the nuclein nitrogen is soluble (purin bases). At this point an approximate equilibrium has apparently been reached, and after this as autolysis progresses there is only a slow increase in the soluble nuclein nitrogen. Here we find an unexpected fact, that when all visible nuclear structures are lost, three

fourths of the nucleins are still demonstrable. Although nuclein disintegration goes at first hand in hand with karyolysis, it is not completed with the completion of karyolysis.

In vivo, however, the conditions are different, and the nuclein nitrogen may be reduced to a very small percentage by the time karyolysis is complete, the nuclein nitrogen being removed almost as fast as it is liberated. In autolysis *in vivo* diffusion into the surrounding body fluids probably takes place, this apparently being shown by the fact that no soluble purin nitrogen was found in the analysis of the spleen autolyzed *in vivo*.

In the normal spleen about 24 per cent. of the total phosphorus exists in the form of phosphatids. After autolysis *in vitro* only a slight decrease in the phosphatid content has occurred even when pyknosis is marked. However, when karyorrhexis and karyolysis are conspicuous features of the tissue changes, the phosphatid phosphorus has decreased to about 20 per cent. of the total phosphorus, and at the completion of karyolysis the phosphatid phosphorus amounts to about 16 per cent. of the total. Finally, when nuclear destruction is histologically complete, the phosphatid phosphorus has decreased to about 13.6 per cent. of the total phosphorus, or about one half to two thirds the amount contained in the normal spleen.

In vivo when histological changes in the spleen had practically come to an end the phosphatid phosphorus had decreased to about 4 per cent. of the total phosphorus still present, and this percentage would have been far lower if it had been calculated with reference to the total phosphorus originally present in the normal spleen.

In the normal spleen about 40 per cent. of the total phosphorus exists in the coagulable and precipitable (insoluble) tissue materials (nucleins, proteins, and organic phosphorus compounds) other than the phosphatids, which were discussed above. After autolysis *in vitro* when pyknosis is prominent there is practically no decrease in this percentage, but with the appearance of karyorrhexis and karyolysis there is a drop to 31.4 per cent. of the total phosphorus, and at the termination of the lytic process the phosphorus content has fallen to 22 per cent. of the total, and decreases to about 19 per cent. when the nuclear destruction is microscopically complete.

In the normal spleen only about 5.7 per cent. of the total nitrogen is in the water-soluble fraction (in the form of ammonium compounds, amino acids, peptones, purin bases, gelatin, etc.), while about 94.3 per cent. of the nitrogen is in an insoluble, coagulable, or precipitable form (proteins, nucleoproteins, and other insoluble organic nitrogen combinations). After autolysis *in vitro* when pyknosis is prominent some of the nitrogen has been changed to the soluble form, so that 7.4 per cent. is soluble. When karyorrhexis and karyolysis are conspicuous the water-soluble nitrogen has markedly increased, so that 26.5 per cent. of the total nitrogen is in this form. The increase, however, is due mainly to amino acids, peptones, and simple nitrogen derivatives, as the part precipitated by tannic acid shows practically no increase. When karyolysis is nearly complete, the soluble nitrogen has increased to 28.6 per cent. of the total nitrogen, and after the microscopical tissue changes have practically ceased the soluble nitrogen amounts to 30.3 per cent., leaving about 69.7 per cent. of the total nitrogen in the insoluble form (precipitable and coagulable by alcohol). However, with autolysis *in vivo* diffusion tends to keep the soluble nitrogen down to about the normal percentage, so that when microscopical tissue disintegration is practically at an end there is but 5.7 per cent. of the total nitrogen in the soluble form, this amount being identical with that in the normal spleen.

SUMMARY.

The most marked chemical changes in a disintegrating spleen are noticed during the period when karyorrhexis and karyolysis are the characteristic features. When pyknosis alone is prominent almost all of the several constituents are in the coagulable or precipitable fraction, just as they are in the original normal tissue. After karyolysis is practically complete the coagulable and precipitable elements of the tissue still gradually decrease in amount, but the rate is far below that occurring during karyorrhexis and karyolysis. Moreover, even when microscopical nuclear disintegration is practically at an end, the chemical process of disintegration is still going on, but very slowly. Finally, when nuclear structures can no longer be seen in the spleen, about 72 per cent. of the nuclein

nitrogen, about 50 per cent. of the original insoluble phosphorus, and 70 per cent. of the original insoluble nitrogen, are still in the insoluble form (precipitable and coagulable by alcohol), while about two thirds of the phosphatid phosphorus remains in complex organic combinations.

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EXPLANATION OF PLATES.

Plate 62.

Figs. 1 and 2. Dog spleen after autolysis for seventeen hours. Microscopically pyknosis is prominent and there is some nuclear fragmentation. Practically all the nuclein nitrogen (about 2.6 per cent. of the total nitrogen) is still in the insoluble and coagulable form, with possibly a trace that is soluble. The phosphorus in the form of phosphatids has, however, suffered a slight decrease in amount, being now about 23.6 per cent. of the total phosphorus. The water-soluble phosphorus has increased slightly (part of this being derived from the

phosphatid fraction) and is now 34 per cent. of the total. The insoluble phosphorus remains about the same, 40.8 per cent. of the total. The water-soluble nitrogen has increased slightly over normal, being 7.4 per cent. of the total (a difference of 1.7 per cent., while the insoluble nitrogen has suffered a corresponding decrease, being now 92.6 per cent. of the total nitrogen, a difference of 1.7 per cent.

PLATE 63.

FIGS. 3 and 4. Dog spleen after autolysis for thirty hours. Microscopically karyorrhexis and karyolysis are conspicuous, especially in the pulp, and 9.7 per cent. of the nuclein nitrogen has become soluble, that is, 0.3 per cent. of the total nitrogen is in the form of soluble purin nitrogen, while 90.3 per cent. of the purin nitrogen, or 2.7 per cent. of the total, is still insoluble. The phosphorus in the phosphatid form has suffered a still further decrease, now amounting to 20.2 per cent. of the total phosphorus, this being a decrease of about 4 per cent. from the normal total phosphorus content. The increase in the water-soluble phosphorus has been more marked, the soluble phosphorus now constituting 47.8 per cent. of the total. In the normal spleen 32 per cent. of the total phosphorus was soluble. The increase is due to additions from the phosphatid and nuclein phosphorus. The decrease in the insoluble phosphorus was also more marked, the percentage dropping to 31.4 per cent. of the total phosphorus, the normal being 40.1 per cent. The water-soluble nitrogen was likewise markedly increased, being 26.5 per cent. of the total, the normal being 5.7 per cent., the increase in the soluble nitrogen being due mainly to the amino acids, peptones, and simpler nitrogen derivatives; while the insoluble nitrogen suffered a corresponding decrease, being 73.5 per cent. of the total nitrogen, the normal being 94.3 per cent.

PLATE 64.

FIGS. 5 and 6. Dog spleen after autolysis for fifty-three hours. Karyolysis has neared completion, only shadows of basophilic nuclear material being left. Of the nuclein nitrogen 27.4 per cent. has become soluble, while 72.6 per cent. is still insoluble. The phosphorus in the phosphatid form has decreased still further in amount, being now only 16 per cent. of the total phosphorus. Partly as a result of this and partly because of the addition of nuclein phosphorus, the water-soluble phosphorus increase is again evident, now amounting to 57.7 per cent. of the total phosphorus. The insoluble phosphorus has decreased to 22 per cent. of the total phosphorus. The water-soluble nitrogen has increased but slightly since the first thirty hours of autolysis, and is now 28.6 per cent. of the total. The insoluble nitrogen has likewise decreased at a slower rate, being now 71.4 per cent. of the total nitrogen. Apparently a point of slow change has now been reached.

PLATE 65.

FIGS. 7 and 8. Dog spleen after autolysis for six days (144 hours). Nuclear destruction is now complete as far as histological changes are concerned, and the other tissue changes have practically reached a standstill. Of the nuclein nitrogen 28 per cent. has become soluble, while 72 per cent. is still insoluble in

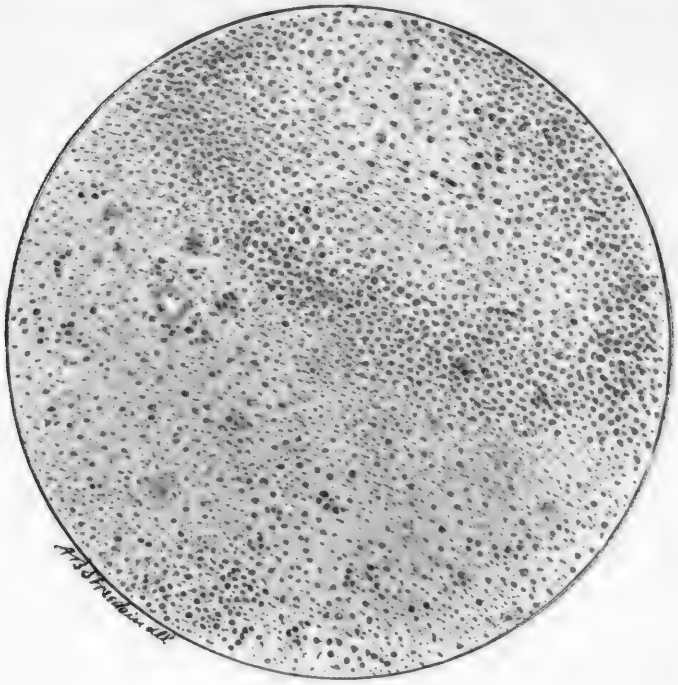

FIG. 1.

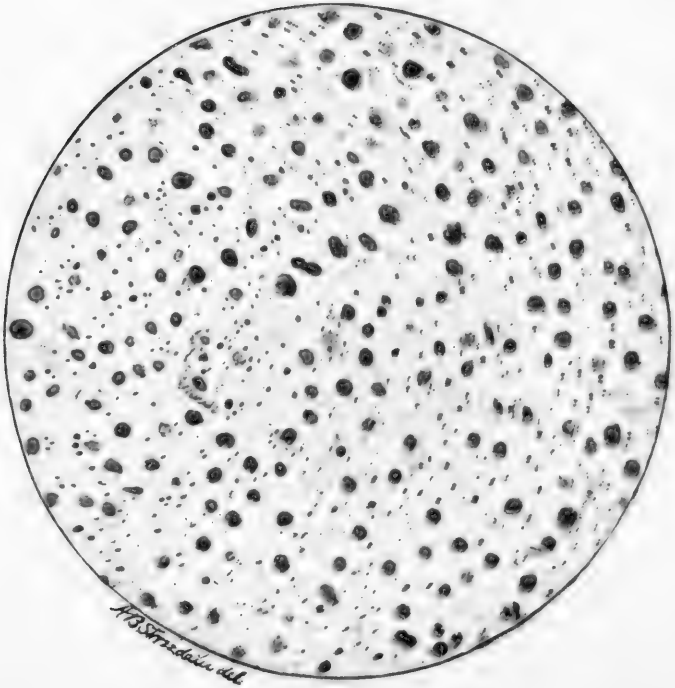

FIG. 2.

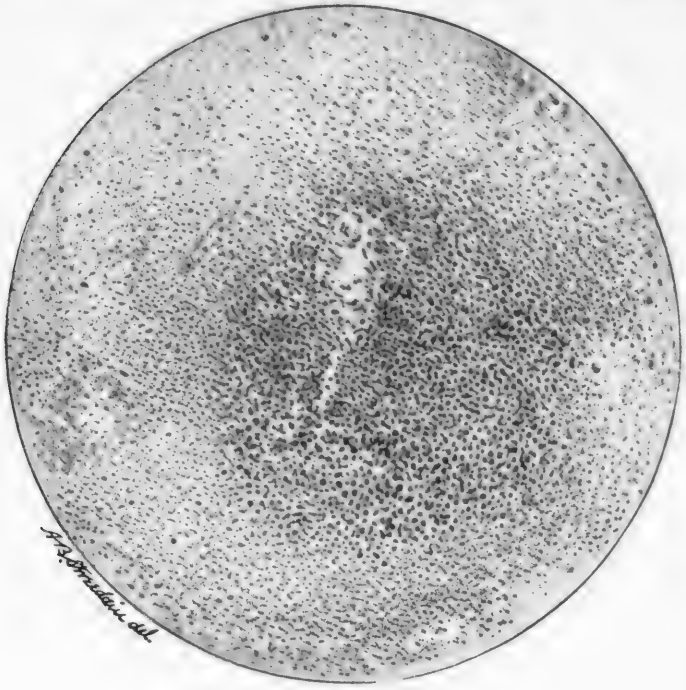

FIG. 3.

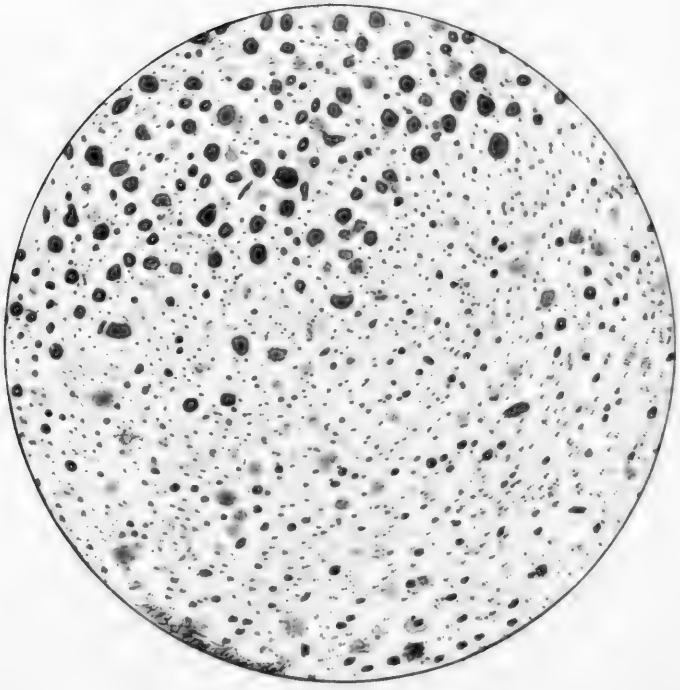

FIG. 4.

FIG. 5.

FIG. 6.

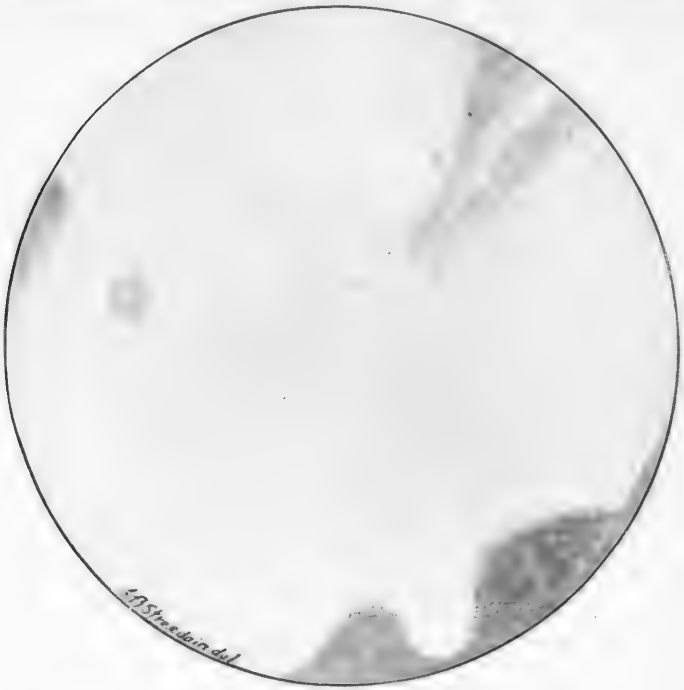

FIG. 7.

FIG. 8.

spite of the loss of all visible nuclear structures. The phosphorus in the phosphatid form has decreased to about half the normal amount, leaving only about 13.6 per cent. of the total phosphorus in this form. The water-soluble phosphorus has increased, now being 67 per cent. of the total, or practically double the amount in the normal spleen. The insoluble phosphorus has decreased to 18.8 per cent. of the total phosphorus, this percentage being about half the normal content, which indicates that the phosphatid phosphorus is set free more rapidly than the nuclein phosphorus, only a slight increase in the water-soluble nitrogen having been noted after autolysis for fifty-three hours. The water-soluble nitrogen is now 30.3 per cent. of the total nitrogen, which is a fivefold increase over the normal. The insoluble nitrogen has slowly decreased, now being 69.7 per cent. of the total nitrogen, a decrease from the normal of about 30 per cent.

The microscopical examination of another dog spleen that had been autolyzed *in vivo* for two days resembled very closely spleen H (figures 7 and 8). Chemically, however, there were characteristic differences. Soluble nuclein nitrogen was absent and there was only 0.3 per cent. of nuclein nitrogen in the insoluble form. The phosphorus in the phosphatid form had decreased markedly in amount, only about 4 per cent. of the total phosphorus being left, and this total had markedly decreased. The water-soluble phosphorus was about the same, or a little more than normally, being 38.86 per cent. of the total phosphorus still present, but compared with the normal spleen content the water-soluble phosphorus had actually decreased one fourth in amount. The insoluble phosphorus constituted a greater percentage than normally of the total phosphorus still present, being 54 per cent., although it had actually decreased to about one third or one fourth of the amount present in the normal spleen. The percentage of the water-soluble nitrogen was about normal, being 5.7 per cent. The percentage was low because the soluble nitrogen was removed as rapidly as it was formed. Of the entire nitrogen of this spleen 94.3 per cent. was still insoluble.

The rate of autolysis *in vivo* was thus shown to be much more rapid than that *in vitro* at room temperature, but the diffusion of the soluble products of autolysis prevented their accumulation in the necrotic spleen tissue *in vivo*. Moreover, the disintegration of nucleins was much more extensive in the autolysis *in vivo* than in that *in vitro*, about six sevenths of the nuclein nitrogen having disappeared.

COMPLETE BALANCE STUDIES OF NITROGEN, SULPHUR, PHOSPHORUS, CALCIUM, AND MAGNESIUM IN INTESTINAL INFANTILISM.*

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PLATES 66–68.

Certain preliminary investigations, published[1] and unpublished, suggested that there was probably a disturbance of the proper balance between the intake and output of material in the cases to which Herter[2] has applied the name intestinal infantilism. To investigate this possibility, complete balance observations were carried out in which the total intake in the food and output in the urine and feces of nitrogen, sulphur, phosphorus, calcium, and magnesium for periods of six days were determined. These observations were upon the two cases of infantilism described in previous papers. As a control on these two cases, a normal child was similarly studied. The diet was a generous, unrestricted one, only non-homogeneous foods being excluded. All the food was weighed and analyzed as usual. The technique employed in carrying on these investigations was essentially that described in detail in earlier papers by one of us.[3]

The results are given in tables I, II, and III. The methods of analysis and the amounts used for analysis are shown in table IV.

Considerations similar to those in an earlier paper[4] indicate that the probable errors in the figures for the balances are within the following limits: Nitrogen, ± 0.2 of a gram; sulphur, ± 0.05 of a

*Received for publication, February 24, 1912.

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TABLE I.

Boy, F. S., with Infantilism. Weight at Beginning of Experiment, 14.1 Kilograms; Weight at End, 14.5 Kilograms.

	Day.	Nitrogen.	Calcium oxid.	Magnesium oxid.	Phosphorus pentoxid.
Urine	1	2.75	0.010	0.005	0.512
	2	2.76	0.016	0.070	0.520
	3	3.02	0.021	0.086	0.572
	4	2.75	0.012	0.074	0.497
	5	2.51	0.009	0.054	0.398
	6	2.32	0.040	0.111	0.211
	Total	16.11	0.108	0.400	2.710
	Total weight.				
Feces	1 ..... 29.6	1.707	1.965	0.213	0.855
	2 ..... 14.32	0.868	0.750	0.009	0.767
	3 ..... 58.5	3.532	2.744	0.579	2.495
	4 ..... 40.35	2.840	1.733	0.339	1.077
	5 ..... 52.55	2.286	1.618	0.331	1.072
	6 ..... 35.75	2.288	1.451	0.229	1.073
	Total 231.1	13.52	10.26	1.700	7.34
Total excretion		29.63	10.37	2.10	10.25
Total in food		35.64	6.35	2.43	11.91
Retention		6.01	....	0.33	1.66
Loss		....	4.02	....	....
Per cent. retained		16.9	....	1.4	13.9
Per cent. lost		....	63.3	....	....

gram; calcium oxid, ± 0.04 of a gram; magnesium oxid, ± 0.02 of a gram; phosphorus pentoxid, ± 0.07 of a gram.

Calcium.—Examination of the balances showed a striking loss of calcium by F. S., in spite of the fact that everything else was retained. In the case of F. H., magnesium as well as calcium was lost. Sulphur and nitrogen were retained. The results in these two cases were in striking contrast to those of the normal boy in which all the elements were retained in proportion to the amounts in which they were metabolized.

In the two cases of infantilism, the calcium excretion in the urine was almost negligible, the amount excreted in a week being less than the amount normally excreted in a day. The amount of calcium in the urine was only 1 to 2 per cent. of the amount in the feces, this being in marked contrast to the normal case where it was 20 per cent. Practically all the calcium was in the feces. In the

TABLE II.

Boy, F. H., with Infantilism. Weight at Beginning of Experiment, 11.8 Kilograms; Weight at End, 12.2 Kilograms.

Day.		Nitrogen.	Sulphur.	Calcium oxid.	Magnesium oxid.
Urine	1	5.68	0.432	0.050	0.061
	2	5.44	0.412	0.017	0.058
	3	5.27	0.392	0.014	0.063
	4	5.62	0.428	0.028	0.068
	5	5.87	0.452	0.018	0.073
	6	5.56	0.424	0.029	0.067
	Total	33.44	2.540	0.156	0.390
	Total weight.				
Feces	1 17.4	0.789	0.089	1.583	0.300
	2 21.3	0.989	0.117	1.632	0.337
	3 17.5	0.836	0.099	1.279	0.287
	4 18.7	0.899	0.102	1.265	0.308
	5 22.7	1.016	0.154	1.577	0.336
	6 19.7	0.875	0.114	1.449	0.274
	Total 117.3	5.404	0.675	8.785	1.842
Total excretion		38.84	3.216	8.941	2.231
Total in food		48.77	4.110	8.820	1.968
Retention		9.93	0.894	....	....
Loss		....	....	0.121	0.263
Per cent. retained		20.3	21.8	....	....
Per cent. lost		....	....	1.4	1.3

case of F. H., there was as much calcium in the feces as in the food, and in the case of F. S., 60 per cent. more than in the food.

Magnesium.—The magnesium, like the calcium, was low in the urine and high in the feces. In the urine of the normal boy, the quantity of magnesium was 56 per cent. of that in his feces, while in the urine of the two patients with infantilism, it was only about 20 per cent. of that in the feces. The feces of the normal boy contained about half as much magnesium as was present in his food; whereas the feces of F. S. contained more than two thirds as much, and the quantity in the feces of F. H. nearly equalled that in his food.

Nitrogen.—In the stools the amount of nitrogen was high. In F. S. it was over two grams per day. The absolute amount in F. H. was not greater than in the normal boy, but it was nearly twice as great in proportion, since he was receiving only about half as much per day in his food. If we compare the amount in the feces with

TABLE III.

Normal boy, W. McC. Weight at Beginning of Experiment, 27 Kilograms; Weight at End, 27.2 Kilograms.

Day.		Nitrogen.	Sulphur.	Calcium oxid	Magnesium oxid.	Phosphorus pentoxid.
Urine	1.....	10.43	0.729	0.374	0.153	2.34
	2.....	10.65	0.744	0.406	0.139	2.22
	3.....	10.35	0.717	0.373	0.154	2.23
	4.....	11.07	0.832	0.348	0.152	2.06
	5.....	11.35	0.808	0.332	0.157	2.58
	6.....	11.12	0.810	0.390	0.154	2.68
	Total...	64.97	4.640	2.223	0.909	14.11
	Dry feces					
Feces	1 ...22.8	1.232	0.133	1.518	0.267	0.686
	2 ...19.0	0.997	0.085	1.846	0.263	0.718
	3 ...20.9	1.165	0.115	1.784	0.288	0.768
	4 ...23.5	1.173	0.113	2.084	0.301	0.839
	5 ...15.3	0.811	0.063	1.318	0.220	0.526
	6 ...21.1	1.110	0.073	1.643	0.279	0.856
	Total 122.6	6.488	0.582	10.193	1.618	4.393
Total excretion .		71.46	5.222	12.42	2.53	18.50
Total in food ...		86.10	6.138	14.988	3.263	24.709
Retention.....		14.64	0.916	2.57	0.73	6.20
Per cent. retained.....		17.0	14.9	17.1	22.4	25.0

TABLE IV.

		Nitrogen.	Calcium oxid and magnesium oxid.	Sulphur.	Phosphorus pentoxid.
Amounts used for analysis	urine.....	5 c.c.	200 c.c.	50 c.c.	50 c.c.
	food.....	1.0000 gm.	10.0000 gm.	2.0000 gm.	2.0000 gm.
	feces.....	1.0000 gm.	1.0000 gm.	2.0000 gm.	1.0000 gm.
Method of analysis.....		Kjeldahl	McCrudden[5]	Folin[6]	Neumann[7]

that in the food, we see that it was 38 per cent. in the case of F. S., 11 per cent. in the case of F. H., and only 7.5 per cent. in the case of the normal boy.

In the urine, the fraction of the nitrogen of the food that appeared was smaller than normally, being 45 per cent. in the case of F. S., 67 per cent. in the case of F. H., and 91 per cent. in the case of the normal boy.

[5] McCrudden, *Jour. Biol. Chem.*, 1911, x, 187.

[6] Folin, *Jour. Biol. Chem.*, 1905–6, i, 131.

[7] Neumann, *Ztschr. f. physiol. Chem.*, 1902, xxxvii, 131; Neumann and Meinertz, *idem*, 1904–5, xliii, 37.

The amount of nitrogen in the feces was brought out particularly well by the high ratio which the nitrogen of the feces bore to that of the urine. This was 84: 100 in the case of F. S., 16: 100 in the case of F. H., and only 10: 100 in the case of the normal boy.

Sulphur.—The figures for sulphur, which, like nitrogen, is a measure of protein metabolism, show changes similar to those for nitrogen. The sulphur was not determined in the case of F. S., but in that of F. H. the sulphur excretion in the feces was more marked than that of the nitrogen. The figures are as follows:

	Sulphur proportion: feces to food.	Sulphur proportion: urine to food.	Sulphur proportion: feces to urine.
F. H.....	16.5	62.0	27.0
Normal boy.....	9.5	76.0	12.5

Furthermore, even the absolute amount of sulphur in the feces in F. H. was greater than in the case of the normal boy, in spite of the fact that F. H. was much smaller and had less sulphur in his food.

Phosphorus.—The excretion of phosphates in the stools in infantilism was larger and more marked even than that of calcium, magnesium, nitrogen, and sulphur had been. In the feces of F. S., there was nearly three times as much phosphate as in his urine, while in the urine of the normal boy, there was over three times as much phosphate as in his stools. In the feces of the normal boy, there was only about one sixth as much phosphate as in his food, while in the feces of F. S. there was over three fifths as much. There was nearly double as much phosphate in the feces of F. S. as in the feces of the normal boy, although the latter had over twice as much phosphorus in his food.

The daily weight of the dry feces was large, being especially so in the case of F. S., where it averaged 3.9 grams per day. The weight of the feces of F. H. was also large in proportion to his size and the amount of food he took.

DISCUSSION.

To summarize briefly, the quantities of nitrogen, sulphur, phosphates, calcium, and magnesium in the feces were high, and the quantities in the urine were low. As an explanation of this condi-

tion, we might think of decreased absorption or increased excretion. If the disturbance affected only the calcium, we might consider increased excretion as a possible cause, but the other elements, especially the sulphur and nitrogen, are not generally excreted in any considerable quantity into the intestines, so it seems probable that lack of absorption must be responsible for the large amounts of these elements.

The disturbance of the calcium metabolism was more marked than that of the other elements. In the case of F. S., the ratio of the calcium to the phosphates in the urine was 4:100, and in the normal boy it was 16:100. The ratio of calcium to magnesium in the urine was 27:100 in the case of F. S., 40:100 in the case of F. H., and about 244:100 in the case of the normal boy. The fact that the excretion of calcium was considerably greater than normally in the two cases of infantilism, when taken in connection with the fact that in one of the cases there was actually a greater amount of calcium in the feces than in the food, must mean that in the case of calcium, in addition to the lack of absorption, there was also an increased excretion through the intestines.

The great loss of calcium may account for the fragility of the bones that was noted, for this led in the case of F. S. to two fractures from very slight causes, these fractures occurring within a few months of each other. X-ray examinations of the skeletal system showed disturbances in accord with these results. The epiphyses throughout were ununited, some of the smaller bones, especially those of the wrist, were missing or uncalcified, and the cortex of the long bones was found to be exceedingly thin, both in absolute figures and in comparison with the diameter of the bone. Examples of these conditions are seen in figures 1 and 2. In figure 1, the right wrist of F. S. is shown, while in figure 2 the bones of the lower part of the leg are seen. By way of comparison, in figure 3 are pictured the leg bones of a frail, eight year old girl of the same weight as F. S., who is an eleven year old boy. There is, in fact, a condition of osteoporosis.

These results are in accord with the facts already pointed out by one of us in various papers on the subject of osteomalacia; namely that this condition should be regarded, not as a distinct disease, but

as a symptom following excessive calcium catabolism, which may be brought about by a variety of different conditions.[8]

The results of the present paper have suggested a number of interesting points which are at present under investigation and are, for this reason, reserved for future papers.

SUMMARY.

In two cases of infantilism there was poor absorption from the intestines of nitrogen, sulphur, phosphorus, calcium, and magnesium.

The excretion of these elements in the urine was low. The excretion of calcium in the urine was so small as to be negligible.

The excretion of calcium into the intestines was increased.

The complete balance experiments showed that while other elements were retained, calcium was lost.

EXPLANATION OF PLATES.

PLATE 66.

FIG. 1. X-ray photograph of the hands of F. H. (infantilism). Some of the carpal bones are missing or uncalcified. The cortex of the bones is seen to be very thin.

PLATE 67.

FIG. 2. X-ray photograph of a leg of F. H. (infantilism). The cortex of the bones is seen to be very thin compared with that of a normal child (figure 3).

PLATE 68.

FIG. 3. X-ray photograph of the legs of a normal child, 9 years old. For comparison with figure 2.

[8] McCrudden, *Arch. Int. Med.*, 1910, v, 596; 1912, ix, 273.

Further evidence on this point is the statement by Trevelyan (Albutt and Rolleston, System of Medicine, 1908, iv, pt. 1, 390) that a condition of osteoporosis is sometimes observed accompanying the increased bone formation in acromegaly.

FIG. 1.

FIG. 2.

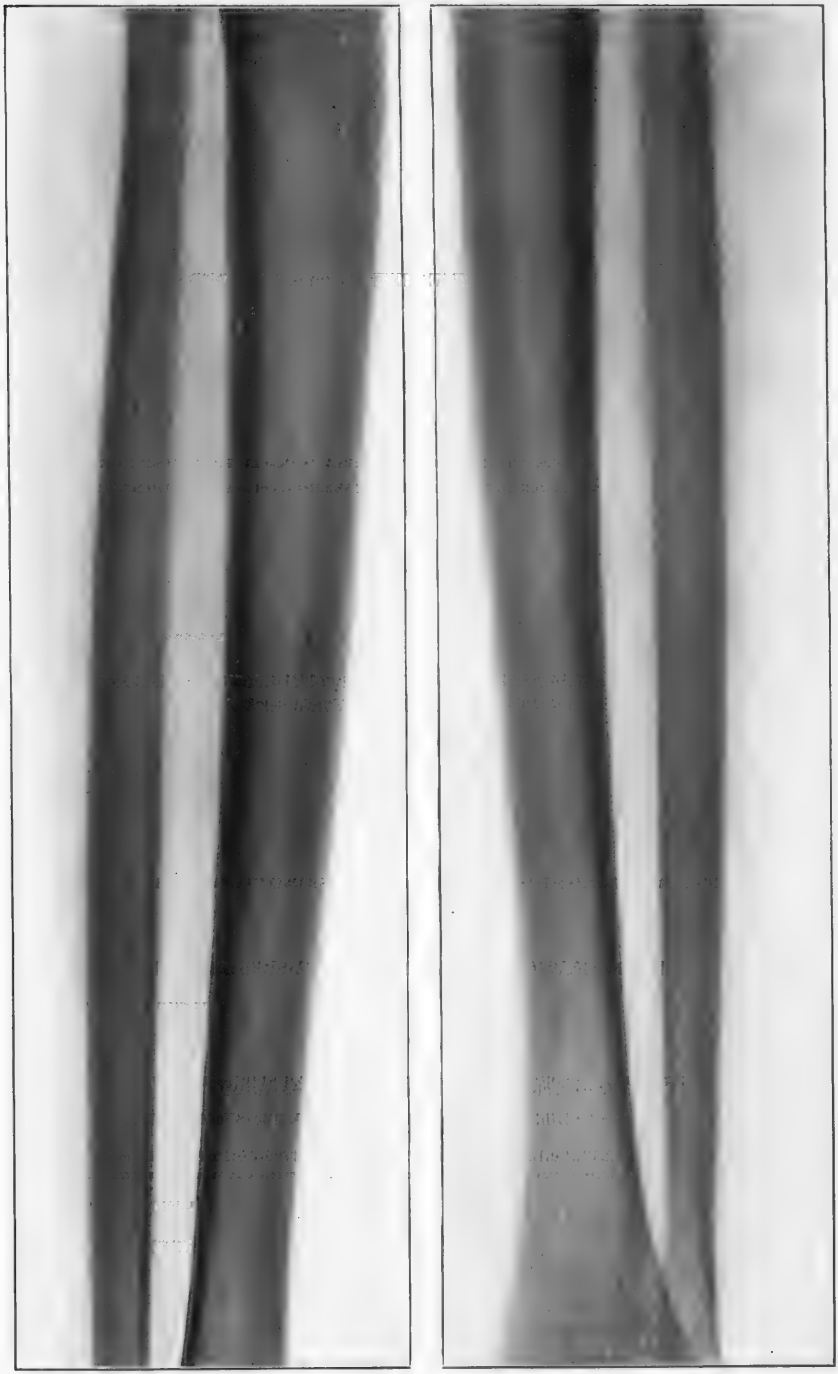

FIG. 3.

THE EFFECT OF FAT AND OF CARBOHYDRATE DIETS ON THE EXCRETION OF CREATIN IN CASES OF RETARDED DEVELOPMENT.*

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Creatin is not a normal constituent of human urine. It occurs in the urine during starvation and inanition, and Cathcart[1] has shown that administration of carbohydrates leads to its disappearance. The disappearance of creatin from the urine of starving rabbits may be brought about in the same way.[2] Likewise in the diabetes in dogs caused by phloridzin, the feeding of carbohydrates leads to the disappearance of creatin.[3] Only carbohydrates have this effect. Fat does not reduce the creatin excretion.

It was observed by the writer that creatin was constantly excreted in certain cases of retarded development, and preliminary observations seemed to indicate that the amount of creatin excreted was greater on a diet rich in carbohydrates than on a diet rich in fats.[4] In view of the great interest and diversity of opinion regarding the metabolism of creatin and creatinin at the present time, and in view also of the bearing which the results might have in the clearing up of the nature of the process in cases of retarded development, the unexpected results of my preliminary observations, showing that on a high carbohydrate diet more creatin may be excreted than on a high fat diet, seemed worthy of further study. The result of comparing the effect of these two diets upon the excretion of creatin in cases of retarded development is given in this paper.

Four patients were used for the observations. Two of them,

* Received for publication, February 24, 1912.

[1] Cathcart, *Jour. Physiol.*, 1909, xxxix, 311.

[2] Mendel and Rose, *Jour. Biol. Chem.*, 1911, x, 213.

[3] Cathcart and Taylor, *Jour. Physiol.*, 1910, xli, 276.

[4] McCrudden, *Jour. Exper. Med.*, 1912, xv, 107.

F. S. and J. P., have been referred to in earlier papers,[5] E. B. was an achondroplasiac dwarf, and M. S. a cretin. The observations on each patient were divided into three periods of four days each: first, a preliminary period during which the food consisted of the usual mixed diet of the hospital; second, a period in which the diet was rich in fats; and third, a period in which the diet was rich in carbohydrates.

In the second period, as large amounts as possible of cream, olive oil, and butter were given; the meat, consisting of young chicken, ham, pork, and bacon, was selected to contain as much fat as possible; and the bread, potatoes, and sugar were reduced to the lowest quantities. The bread was cut thin and was thickly smeared with butter, and the potatoes were mashed and contained as much moisture and butter as possible.

In the third, or carbohydrate period, as much starchy food as possible was given. No butter was used, and instead of cream, separated milk, *i. e.*, milk from which the fat had been almost completely removed, was given. Moreover, the meat selected was as lean as possible. In the diets given below the solid foodstuffs are reported in grams, the liquids in cubic centimeters. The results follow:

Patient I.—F. S., boy, 11 years old. Diagnosis: infantilism of the type of Herter. Height, 106.6 cm. Weight at the beginning of the experiment, 22.1 kilos; weight at the end of the experiment, 22.3 kilos.

I. *Preliminary Period.*—1st to 4th day.

II. *Fat-rich Period. 5th day. Breakfast.*—Bread, 10; cocoa, 125; bacon, 39. *Dinner.*[6]—Cream, 16; bread, 12; sweet potato, 70; mutton chop, 85; olive oil, 30. *Supper.*—Bread, 12; bacon, 37; cream, 40; orange, 92; cocoa, 120.

6th day. Breakfast.—Apple, 57; toast, 15; cream, 45; cocoa, 110; lamb chop, 85; olive oil, 30. *Dinner.*—Bread, 10; bacon, 42; lamb chop, 52; olive oil, 30. *Supper.*—Bread, 12; orange, 72; cream, 50; cocoa, 110; ham, 85; olive oil, 30.

7th day. Breakfast.—Apple, 60; toast, 10; cream, 50; cocoa, 110; bacon, 35; olive oil, 30. *Dinner.*—Bread, 10; orange, 10; cream, 75; mutton chop, 50; olive oil, 30. *Supper.*—Bread, 9; baked apple, 47; cream, 75; cocoa, 110.

8th day. Breakfast.—Apple, 35; toast, 9; cream, 60; cocoa, 110; lamb chop, 47; olive oil, 30. *Dinner.*—Sweet potato, 35; bread, 9; cream, 50; cocoa, 110; lamb chop, 60. *Supper.*—Bread, 9; cream, 65; cocoa, 110; apple, 50; ham, 62.

In addition, during these four days a total of 136 grams of butter and 130 grams of sugar was given.

[5] McCrudden, *loc. cit.;* McCrudden and Fales, *Jour. Exper. Med.*, 1912, xv, 113; McCrudden and Fales, *idem*, 450.

[6] Through an oversight, some banana was given at this meal.

III. *Carbohydrate-rich Period. 9th day. Breakfast.*—Grape fruit, 116; toast, 29; separated milk, 150. *Dinner.*—Rice, 52; beef, 49; beans, 19; biscuit, 11; bread, 18; tapioca and fruit, 60. *Supper.*—Bread, 29; honey, 5; separated milk, 150; meat, 60.

10th day. Breakfast.—Toast, 19; marmalade, 20; grape fruit, 89; separated milk, 150. *Dinner.*—Rice, 73; bread, 24; gelatin, 122; honey, 16; beef, 37. *Supper.*—Bread, 25; jam, 22; separated milk, 150; meat, 89.

11th day. Breakfast.—Grape fruit, 94; toast, 20; marmalade, 22; beefsteak, 67. *Dinner.*—Bread, 20; honey, 15; rice, 75; tapioca and fruit, 115; beefsteak, 92. *Supper.*—Bread, 28; apple, 83; jam, 20; biscuit, 9; meat, 85; separated milk, 85.

12th day. Breakfast.—Orange, 142; toast, 22; marmalade, 20; separated milk, 150. *Dinner.*—Bread, 17; rice, 83; gelatin, 109; honey, 10; steak, 87. *Supper.*—Bread, 19; meat, 65; jam, 20; separated milk, 200.

In addition, a total of 33 grams of sugar was given during this period.

Patient II.—J. P., boy, 17 years old. Diagnosis: infantilism of the type of Herter. Height, 118.2 cm. Weight at the beginning of the experiment, 22.2 kilos; weight at the end, 22.9 kilos.

I. *Preliminary Period.*—1st to 4th day.

II. *Fat-rich Period. 5th day. Breakfast.*—Bread, 10; egg, 120. *Dinner.*[1]—Cream, 16; bread, 12; potato, 70; mutton chop, 82; olive oil, 30. *Supper.*—Bread, 12; cream, 50; sardines, 7; orange, 85; cocoa, 120.

6th day. Breakfast.—Apple, 44; egg, 90; toast, 15; cream, 75; cocoa, 100; olive oil, 30. *Dinner.*—Lamb chop, 38; potato, 40; bread, 10; olive oil, 30. *Supper.*—Bread, 12; orange, 72; onion, 10; salmon, 20; olive oil, 30.

7th day. Breakfast.—Apple, 50; toast, 10; cream, 50; cocoa, 110; egg, 92; lamb chop, 18; olive oil, 30. *Dinner.*—Potato, 18; bread, 10; orange, 10; cream, 75; chicken, 73; olive oil, 30. *Supper.*—Bread, 9; egg, 45; cream, 75; cocoa, 110; baked apple, 47.

8th day. Breakfast.—Apple, 35; toast, 9; cream, 60; cocoa, 110; egg, 25; olive oil, 15. *Dinner.*—Sweet potato, 35; bread, 9; cream, 50; cocoa, 110; lamb chop, 60. *Supper.*—Bread, 9; apple, 30; cream, 65; cocoa, 110; lamb chop, 34; olive oil, 30.

In addition, a total of 215 grams of butter and 96 grams of sugar was given during this period.

III. *Carbohydrate-rich Period. 9th day. Breakfast.*—Orange, 90; toast, 29; separated milk, 150. *Dinner.*—Bread, 18; beans, 39; potato, 49; chicken, 38; biscuit, 9; separated milk, 150. *Supper.*—Bread, 38; tomato, 42; honey, 5; separated milk, 150; scraped beef, 60.

10th day. Breakfast.—Toast, 28; marmalade, 6; orange, 71; separated milk, 150. *Dinner.*—Bread, 22; potato, 50; honey, 18; gelatin, 122; chicken, 60; gravy, 15. *Supper.*—Bread, 25; jam, 17; separated milk, 150; meat, 80.

11th day. Breakfast.—Apple, 60; oatmeal, 100; separated milk, 150; toast, 7; steak, 35. *Dinner.*—Chicken, 42; steak, 32; bread, 19; separated milk, 150; tapioca and fruit, 100. *Supper.*—Bread, 27; jam, 20; apple sauce, 83; biscuit, 9; meat, 97; separated milk, 150.

[1] Through an oversight, some banana was given at this meal.

12th day. Breakfast.—Orange, 132; toast, 26; honey, 15; separated milk, 150. *Dinner.*—Bread, 3; onion, 8; chicken, 12; potato, 19; gelatin, 50; bread, 20; honey, 11. *Supper.*—Bread, 37; steak, 110; tomato, 55; jam, 18; separated milk, 250.

In addition, a total of 65 grams of sugar was given during this period.

Patient III.—E. B., boy, 4 years old. Diagnosis: achondroplasia. Height, 78 cm. Weight at the beginning of the experiment, 14.8 kilos; weight at the end, 14.5 kilos.

I. *Preliminary Period.*—1st to 4th day.

II. *Fat-rich Period. 5th day. Breakfast.*—Baked apple, 70; egg, 82; bread, 8; cream, 100; cocoa, 70. *Dinner.*—Chicken, 54; potato, 30; bread, 8; cream, 60. *Supper.*—Biscuit, 8; grapes, 25; egg, 70; bread, 8; cream, 75; cocoa, 70.

6th day. Breakfast.—Orange juice, 100; bacon, 20; egg, 75; toast, 9; cream, 75; cocoa, 70. *Dinner.*—Bread, 9; chicken, 70; potato, 25; jelly, 20; cream, —. *Supper.*—Bread, 8; cream, 75; cocoa, 70; egg, 57.

7th day. Breakfast.—Egg, 85; toast, 8; orange juice, 85; cream, 65; cocoa, 70. *Dinner.*—Bread, 7; pork, 62; potato, 20; water, 200. *Supper.*—Bread, 8; pork, 100; cream, 65; cocoa, 90; apple, 50.

8th day. Breakfast.—Bacon, 15; egg, 50; cream, 75; cocoa, 70; toast, 9; orange juice, 100. *Dinner.*—Bread, 8; potato, 20; chicken, 35. *Supper.*—Bread, 8; egg, 85; biscuit, 5; cream, 95; cocoa, 110.

In addition, during the whole period a total of 113 grams of sugar and 118 grams of butter was used.

III. *Carbohydrate-rich Period. 9th day. Breakfast.*—Toast, 27; honey, 8; oatmeal, 100. *Dinner.*—Meat, 50; apple, 100; potato, 50. *Supper.*—Bread, 12; meat, 50; beans, 70; honey, 10; biscuit, 7.

10th day. Breakfast.—Cereal, 100; orange juice, 110; toast, 14; separated milk, 150. *Dinner.*—Bread, 15; potato, 70; scraped beef, 50; jelly, 12. *Supper.*—Meat, 50; bread, 11; apple, 54; separated milk, 150; biscuit, 22.

11th day. Breakfast.—Toast, 23; orange, 120; honey, 8; scraped beef, 45; separated milk, 150. *Dinner.*—Beans, 50; potato, 50; meat, 50; water, 200. *Supper.*—Bread, 17; jelly, 8; apple sauce, 50; potato, 50; roast beef, 29; separated milk, 200.

12th day. Breakfast.—Orange, 120; oatmeal, 170; toast, 10; honey, 5; separated milk, 50. *Dinner.*—Potato, 40; meat, 50; rice, 59; water, 200. *Supper.*—Scraped beef, 62; bread, 15; fruit, 67; separated milk, 150.

In addition, 132 grams of sugar were used during this period.

Patient IV.—M. S., girl, 8 years old. Diagnosis: cretinism. Height, 90 cm. Weight at the beginning of the experiment, 16.9 kilos; weight at the end, 17.3 kilos.

I. *Preliminary Period.*—1st to 4th day.

II. *Fat-rich Period. 5th day. Breakfast.*—Bacon, 7; egg, 55; toast, 7; orange juice, 85; cream, 50. *Dinner.*—Bacon, 16; potato, 16; bread, 4; apple, 50; cream, 50. *Supper.*—Egg, 50; cream, 50; bread, 7; apple sauce, 35.

6th day. Breakfast.—Bacon, 21; toast, 4; egg, 40; cream, 70; orange juice,

100. *Dinner.*—Chicken, 45; potato, 30; apple sauce, 39; cream, 50. *Supper.*—Egg, 40; bread, 5; cream, 75; prunes, 25.

7th day. Breakfast.—Orange juice, 110; egg, 40; cream, 80; toast, 6. *Dinner.*—Lamb chop, 53; bread, 5; apple sauce, 40; cream, 55. *Supper.*—Bread, 6; egg, 35; cream, 80.

8th day. Breakfast.—Orange juice, 110; egg, 30; bacon, 38; bread, 3; cream, 60. *Dinner.*—Pork, 55; apple sauce, 50; bread, 4; potato, 25. *Supper.*—Egg, 40, cream, 75; bread, 4.

In addition, a total of 27 grams of sugar and 125 grams of butter was used during this period.

III. *Carbohydrate-rich Period. 9th day. Breakfast.*—Cereal, 100; jelly, 25; bread, 15; separated milk, 130. *Dinner.*—Potato, 50; scraped beef, 50; soup, 130; bread, 15. *Supper.*—Bread, 14; jelly, 15; gelatin, 40; separated milk, 130.

10th day. Breakfast.—Oatmeal, 80; separated milk, 150; toast, 17; jelly, 12. *Dinner.*—Rice, 28; beans, 42; scraped beef, 41. *Supper.*—Bread, 20; strawberry jam, 30; separated milk, 150.

11th day. Breakfast.—Orange juice, 100; hominy, 126; separated milk, 150; toast, 12; jelly, 10. *Dinner.*—Tomato, 63; bread, 22; bean soup, 150; meat, 42. *Supper.*—Bread, 22; jam, 23; separated milk, 150.

12th day. Breakfast.—Cream of wheat, 92; separated milk, 155; toast, 22; strawberry jam, 12. *Dinner.*—Bean soup, 150; roast beef, 17; spinach, 60; potato, 62; bread, 19. *Supper.*—Bread, 22; separated milk, 250; apple sauce, 72.

13th day. Breakfast.—Apple, 52; oatmeal, 96; separated milk, 150; jelly, 20; toast, 15. *Dinner.*—Chicken, 43; potato, 50; bread, 22. *Supper.*—Bread, 28; separated milk, 210; jam, 10.

In addition, during this period there was given a total of 99 grams of sugar.

RESULTS.

TABLE I.

Patient, F. S.

Day.	Period.	Total nitrogen.	Creatinin plus creatin.	Creatinin.	Average creatinin.	Creatin.	Average creatin.
1	Preliminary	4.95	0.530	0.298	0.283	0.232	0.221
2		4.56	0.443	0.270		0.173	
3		4.34	0.455	0.312		0.143	
4		4.95	0.587	0.250		0.337	
5	Fat-rich diet	5.04	0.580	0.312	0.306	0.268	0.362
6		5.02	0.765	0.338		0.427	
7		4.03	0.572	0.253		0.319	
8		4.31	0.750	0.320		0.430	
9	Carbohydrate-rich diet	5.73	0.666	0.286	0.304	0.380	0.629
10		6.42	0.799	0.261		0.538	
11		9.94	1.312	0.328		0.984	
12		8.26	0.952	0.340		0.612	

TABLE II.

Patient, J. P.

Day.	Period.	Total nitrogen.	Creatinin plus creatin.	Creatinin.	Average creatinin.	Creatin.	Average creatin.
1	Preliminary	5.34	0.502	0.354	0.375	0.148	0.226
2		5.48	0.545	0.373		0.172	
3		5.90	0.635	0.405		0.230	
4		6.76	0.723	0.368		0.355	
5	Fat-rich diet	5.77	0.567	0.354	0.326	0.213	0.229
6		4.77	0.536	0.289		0.247	
7		5.23	0.596	0.331		0.265	
8		3.61	0.521	0.329		0.192	
9	Carbohydrate-rich diet	5.05	0.723	0.405	0.319	0.318	0.491
10		6.43	0.800	0.415		0.385	
11		9.26	1.117	0.424		0.693	
12		8.42	0.999	0.432		0.567	

TABLE III.

Patient, E. B.

Day.	Period.	Total nitrogen.	Creatinin plus creatin.	Creatinin.	Average creatinin.	Creatin.	Average creatin.
1	Preliminary	5.68	0.444	0.222	0.224	0.222	0.411
2		3.62	0.389	0.214		0.175	
3		4.70	0.440	0.256		0.184	
4		3.40	0.370	0.203		0.167	
5	Fat-rich diet	4.06	0.381	0.202	0.189	0.179	0.342
6		5.26	0.381	0.202		0.179	
7		3.76	0.289	0.184		0.105	
8		3.85	0.316	0.167		0.149	
9	Carbohydrate-rich diet	4.76	0.491	0.263	0.239	0.228	0.618
10		5.61	0.611	0.212		0.399	
11		6.57	0.694	0.243		0.451	
12		6.49	0.677	0.237		0.440	

DISCUSSION.

The data show that the creatin excretion is higher on a carbohydrate-rich diet than on a fat-rich diet. The creatin excretion in the third period was increased over that in the second period by 74 per cent. in the case of F. S., by 115 per cent. in the case of J. P., by 81 per cent. in the case of E. B., and by 40 per cent. in the case of M. S. On the average, this was an increase of about 76 per cent. The creatin excretion in the case of M. S. was so variable from day to day that perhaps too much emphasis ought not to be laid on the

TABLE IV.

Patient, M. S.

Day.	Period.	Total nitrogen.	Creatinin plus creatin.	Creatinin.	Average creatinin.	Creatin.	Average creatin.
1	Preliminary	3.41	0.378	0.322	0.339	0.057	0.058
2		3.83	0.455	0.370		0.085	
3		3.80	0.316	0.316		0.000	
4		4.29	0.437	0.347		0.090	
5	Fat-rich diet	3.24	0.312	0.312	0.294	0.000	0.055
6		3.70	0.390	0.312		0.078	
7		4.96	0.444	0.356		0.088	
8		3.18	0.277	0.217		0.060	
9[8]	Carbohydrate-rich diet	3.86	0.449	0.339	0.330	0.111	0.077
10		3.01	0.380	0.293		0.087	
11		3.77	0.382	0.332		0.051	
12		4.07	0.437	0.372		0.065	
13		3.99	0.398	0.315		0.082	

fact that its average was higher during the third period. At the same time, the presence of any creatin at all in the urine on a diet rich in carbohydrates is surprising. But in the other three cases, the increase was striking, the average excretion of creatin being about double during the carbohydrate-rich period.

The creatinin excretion was, on the whole, very constant and was not affected by the diet.

The results presented here are different from those heretofore obtained in studies of creatinuria, and show the inadequacy of the explanation that has previously been given for the appearance of creatin in the urine in conditions such as diabetes and starvation. According to this explanation, when the direct supplies of carbohydrate are cut off in these conditions, there is an increased catabolism of protein of the body tissues in order to form the necessary amounts of carbohydrate, and this abnormally increased catabolism of body protein is accompanied by the production of abnormal products, such as acetone and creatin. But, as we have seen, after the ingestion of larger amounts of carbohydrates, the creatin does not disappear, but is increased.

Wolf[9] has recently called attention to the fact that he has ob-

[8] A small amount of urine, though probably not more than 1/30 of the whole, was lost on this day, so that the correct figures would be even a little higher than those given.

[9] Wolf, *Jour. Biol. Chem.*, 1912, x, 473.

served creatin in the urine in cases where carbohydrate was present in the diet, but the cases he refers to are those of persons suffering from typhoid fever and pneumonia.[10] In these diseases, there is, due to the infection and the high temperature, an increased destruction of body protein, and this destruction cannot be entirely prevented by the feeding of carbohydrates.

From the fact that there is, on the whole, rather more nitrogen excreted in the third period than in the second, the creatin of the meat in the food will suggest itself as one possible source of the creatin in the urine. But against the view that the creatin comes wholly from the meat are the following objections: (1) in the earlier observations creatin was found in the urine on a meat-free diet;[11] and (2) the amount does not vary with the amount of nitrogen excreted, but depends more on the amount of fat and carbohydrate in the diet, than on the amount of meat. This can be observed in the tables in several instances. It is seen, for example, in table III on the fifth, sixth, and tenth days. On the fifth day the patient had, among other things, 54 grams of chicken and there were 4.06 grams of nitrogen in the urine; on the sixth day the amount of chicken was raised to 70 grams and the nitrogen of the urine rose to 5.26 grams. Yet on both days the amount of creatin excreted was the same. On the ninth and tenth days, the nitrogen excretion was respectively a little below and a little above that of the sixth day; yet the creatin excretion for these days was higher, and on the tenth day it was more than twice as high. Still other examples may be found. These considerations exclude the possibility that either the whole or even a large part of the creatin of the urine depends on variations in the amount of the creatin in the food. But they do not exclude the possibility that the source of some of the urinary creatin is the creatin of the food.

The constancy in the excretion of the creatinin and the great variation in the excretion of the creatin in all these observations lend support to the conception that the metabolism of creatin and that of creatinin are independent of each other. Moreover, these observations are hardly in accord with the view that creatin and creatinin

[10] Ewing and Wolf, *Arch. Int. Med.*, 1909, iv, 330; Wolf and Lambert, *Arch. Int. Med.*, 1910, v, 406.

[11] McCrudden, *loc. cit.*

are easily changed into each other in the organism, and that the sum of the two is of more importance than the amount of either one alone.

The results here presented are of importance with reference to their bearing on creatin and creatinin metabolism, but the physiology of these compounds is not at present well enough understood to enable us to show the bearing of the results on the nature of the processes going on in infantilism. Our findings are of considerable importance in relation to the cases under investigation, if we agree with Folin that creatin is a foodstuff and, when fed, may appear in the urine, provided the state of nutrition of the body is high, and the tissues are saturated with creatin. Rubner[12] has pointed out that two things are concerned in the process of growth: (*a*) the "tendency to grow," which is present only in immature animals and without which no animal can grow, however well nourished it may be; and (*b*) the foodstuffs which furnish the material for growth. Thus far in our investigations we have been unable to determine which of these two factors is at fault in the cases of infantilism of the type of Herter. If, however, Folin's view is confirmed, and the increased excretion of creatin on the carbohydrate-rich diet is taken to mean that the state of nutrition of the body is high and that for this reason a greater fraction of the creatin of the food passes through into the urine, the important conclusion must be drawn that the retarded development in these cases is due, not to an insufficiency in the materials for growth, but to the absence of the "tendency to grow."

Studies of other possibilities suggested by the results of the observations reported in this paper are now under way.

CONCLUSIONS.

In two cases of infantilism of the type of Herter, in one case of achondroplasia, and in one case of cretinism, creatin was constantly present in the urine and was increased in amount when the ingestion of carbohydrates was increased.

[12] Rubner, *Arch. f. Hyg.*, 1908, lxvi, 81.

PURE CULTIVATION OF SPIROCHÆTA REFRINGENS.*

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PLATE 69.

In 1905, in the article in which the discovery of *Treponema pallidum* was announced, Schaudinn and Hoffmann described a coarse spirochæta found in superficial lesions on the genitals. This organism was observed not only in syphilitic, but also in non-syphilitic lesions. It is differentiated from the pallidum by its larger dimensions, its plump shape, its comparatively few, irregular curves, and by its readiness to take various anilin dyes. The Giemsa solution stains it violet red. Schaudinn and Hoffmann named this organism *Spirochæta refringens,* and considered it non-pathogenic.

While the organism frequently comes under observation in examining scrapings for the diagnosis of syphilis, yet our exact knowledge of its biology has been fragmentary for the reason that no one had succeeded in isolating it in pure culture. Unquestionably, certain investigators have obtained a growth of this spirochæta in highly impure cultures, but a study based upon such contaminated material aided little in advancing our knowledge.

In my systematic attempt to secure pure cultures of various spirochætæ, I have succeeded in growing this organism in pure culture.

The strain of *Spirochæta refringens* that I cultivated was derived from the scrapings of a moist condyloma around the anus of a female patient. Under the dark-field microscope, the scrapings showed a moderate number of the refringens. The material was ground in a sterile mortar with sodium citrate solution, and the emulsion thus prepared was inoculated into several tubes of solid culture medium consisting of one part of ascitic fluid, two parts of slightly alkaline agar, and a piece of fresh rabbit kidney. The

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technique employed was the same as that for the cultivation of the pallidum[1] from a human being. After two weeks' incubation at 37° C. an impure culture was obtained. By the same process that was used for the pallidum and for the mouth spirochætæ, the refringens was finally purified.

The Properties of the Pure Culture (Figures 1 to 5).—Spirochæta refringens grows luxuriantly in the deeper part of a tube of ascitic tissue agar, and forms hazy colonies that gradually extend from the deeper parts of the tube to the upper. It is an anaerobic organism and no growth takes place within two centimeters of the surface of the medium. At 37° C., the growth becomes visible on about the fourth day after the inoculation, and proceeds for some weeks. It does not produce any visible changes in the medium, and the culture is free from any odor. Unlike the pallidum, a growth is obtainable in the medium without the addition of fresh tissue, although, like the microdentium, the growth is poorer when the tissue is absent.

In pure culture, *Spirochæta refringens* measures, on the average, about 0.5 to 0.75 of a micron in width, and about 6 to 24 microns in length. Chains of two, three, and four or more individuals may be seen. They are connected with one another by means of a thin filament. The number of curves varies with different individuals, but, as a rule, the middle part of the body is merely wavy, while the two extremities are more regularly and deeply curved. Not infrequently, however, the majority of the spirochætæ in certain cultures are fairly regularly curved throughout their entire length, the distance between two curves measuring about 2 microns. The ends of the organism are pointed, and to one or both of them is usually attached a fine, long, more or less stiff, but elastic projection, which has regular minute curves.

In a culture growing under optimum conditions, the spirochætæ are very motile. Under these conditions the curved flagella-like projections rotate with extraordinary rapidity around their point of attachment to the spirochætæ. While the flagella-like projection is rotating rapidly, the body of the spirochæta may remain motionless, or may be traversed by waves, convex curves becoming concave, and concave curves becoming convex. The waves begin at

[1] Noguchi, *Jour. Exper. Med.*, 1912, xv, 90.

one end and may pass along the entire body, or may stop after having passed over only a few curves. Whenever motility is observed in the body of the spirochæta, the movements are repeated at frequent intervals. In a dead specimen, the curves are much more irregular and are often almost straightened out.

The body of the refringens was thought by Schaudinn to be ribbon-like, but in pure culture I find it to be cylindrical. In the refringens cultivated by me, there is no undulating membrane.

Regarding the mode of multiplication, I am still unable to decide whether or not a longitudinal division occurs before the spirochætæ are found attached end to end in pairs or in chains of several individuals. The ultimate division of these chain forms is transverse.

Pathogenicity.—Several attempts were made to produce lesions in the testicles of rabbits, or in the skin of *Macacus rhesus* monkeys, but thus far without a positive result.

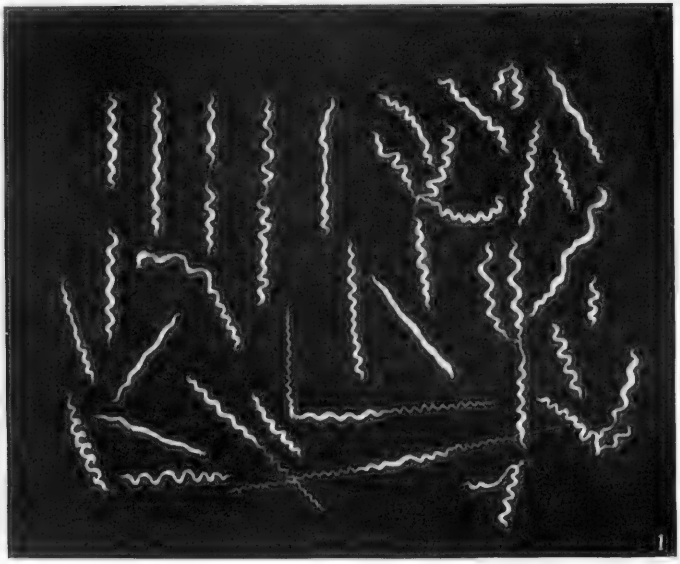
GENERAL CONSIDERATION.

That *Spirochæta refringens* constitutes an independent species, and not a form representing merely a certain stage of *Treponema pallidum,* is proved by the present study and by my published observation on different strains of the pallidum in pure culture. Neither the refringens nor the pallidum lose their characteristics at any stage of cultivation. As a consequence, they may always be distinguished even in cultures.

A few investigators in questioning the evidence for the specificity of *Treponema pallidum* for syphilis have argued that, if the refringens is a form of the pallidum, the latter cannot be specific for syphilis, because the former is found in so many non-syphilitic lesions. As the two organisms are now shown to be different, this argument can no longer be employed.

Spirochæta refringens belongs, doubtless, to the genus *Treponema,* but, like the other members of this genus, it is entirely distinct from the pallidum.

There are still some important problems that await solution by means of pure cultures. Balanitis erosiva circinata is a clinically well defined disease, and in the lesion a large spirochæta resembling the refringens is usually found. Von Prowazek and Hoffmann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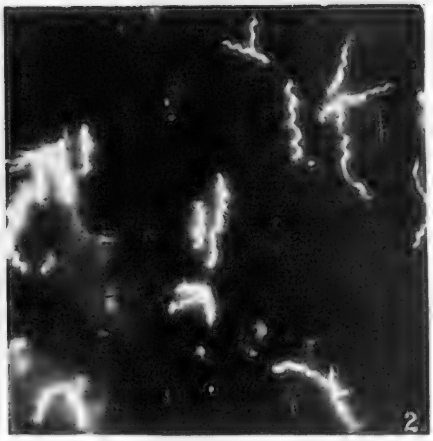
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considered this organism to be its causative agent. Mindful of the fact that the refringens has a wide distribution in the genital region, one naturally seeks a means by which the two morphologically allied forms, the refringens and balanitidis, can be definitely differentiated. This is possible only through the growth of both organisms in pure culture. Unfortunately, I have seen but few cases of this type of balanitis and for this reason I have been unable to attempt seriously its cultivation.

From the point of view of cultivation, another problem of interest is the relation between *Spirochæta buccalis* and the refringens. There is at present much controversy in regard to the identity of these spirochætæ. Some consider them identical, while others regard them as independent varieties. My numerous attempts to isolate the buccalis in pure culture have thus far been fruitless. There seems to be no doubt but that the buccalis is much more difficult to grow on artificial medium than the microdentium or macrodentium. In an impure culture of the buccalis, I was much impressed with the morphological similarity that it bears to the refringens, but the question of the identity of these two spirochætæ requires further study.

SUMMARY.

A strain of *Spirochæta refringens* has been obtained in pure culture, and its morphological and cultural characteristics have been studied. The strain possessed no pathogenicity for rabbits or monkeys. It probably belongs to the genus *Treponema*, but it is easily differentiated from *Treponema pallidum, Treponema microdentium*, and *Treponema macrodentium*. Its relation to *Spirochæta balanitidis* and *Spirochæta buccalis* is still undetermined.

EXPLANATION OF PLATE 69.

FIG. 1. A schematic drawing of *Spirochæta refringens* from pure cultures (dark-field).

FIG. 2. *Spirochæta refringens* from a three weeks' old pure culture in ascitic tissue agar, at 37° C. (dark-field). × 1,100.

FIG. 3. *Spirochæta refringens* from a pure culture seven days old (dark-field). × 1,100.

FIGS. 4 and 5. *Spirochæta refringens* from a pure culture two weeks old (dark-field). × 1,100.

EFFECTS ON TITRATIONS OF INEQUALITY OF SENSITIZATION OF CORPUSCLES.*

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It is well known that corpuscles will absorb many times the amount of specific amboceptor necessary to produce hemolysis, and that such absorption takes place with considerable rapidity. We believe, however, that the importance of these facts in quantitative serum work, as titrations for lytic or complementary power, has not been recognized. The author has frequently noted that duplicate titrations of the same serum gave results that differed beyond reasonable limits of experimental error, and that the reading obtained from a serum titration could be influenced considerably by slight variations in the method of activating the corpuscles. These results appear to be due to the fact that when corpuscles are added to an amboceptor dilution they are not at once evenly distributed through the fluid, and thus, owing to the rapidity with which amboceptor is absorbed, an opportunity is afforded for certain corpuscles to take up more of the amboceptor than others, and unequal sensitization results. If this be the case, it will be seen that slight variations in the method of activating the corpuscles will produce variations in the distribution of amboceptor, and consequently in the hemolysis obtained.

The following complement titrations illustrate the difference in results that are produced by different methods of activating the corpuscles.

Experiment I.—The tubes contained a 1:10 dilution of guinea pig serum in the amounts shown below, with sufficient salt solution to make the final total in each tube 1.25 c.c.

(*A*) To each tube were added 0.25 c.c. of a 1:2,000 dilution of amboceptor (1 unit) and 0.25 c.c. of a 5 per cent. suspension of sheep corpuscles separately.

(*B*) Into 4 c.c. of a 1:2,000 dilution of amboceptor were dropped rapidly, but one drop at a time, 4 c.c. of a 5 per cent. suspension of sheep corpuscles, the receptacle being shaken in the meantime. Of this mixture 0.5 c.c. was added to each tube.

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(*C*) Like *B*, except that the process was reversed, the amboceptor being added to the corpuscles, 0.5 c.c. was added to each tube.

(*D*) Into 4 c.c. of the 5 per cent. suspension of corpuscles were quickly poured 4 c.c. of the amboceptor dilution, and the mixture was immediately shaken. 0.5 c.c. was added to each tube.

The results after incubation for one hour at 37° C. were as follows:

Guinea pig serum, 1:10 dilution.	0.375	0.3	0.25	0.225	0.2	0.175	0.15	0.125	0.1	0.075	0.05	0.025	0
(A)	C.	C.	C.	C.	C.	AC.	AC.	AC.	P.	P.	VS.	VS.	0
(B)	VS.	VS.	VS.	VS.	VS.	VS.	VS.	VS.	VS.	VS.	VS.	VS.	0
(C)	C.	C.	C.	C.	C.	C.	C.	AC.	AC.	P.	VS.	VS.	0
(D)	C.	C.	C.	C.	C.	C.	C.	C.	AC.	P.	VS.	VS.	0

In this and the following experiments, C = complete hemolysis; AC = almost complete hemolysis; P = partial hemolysis; S = slight hemolysis; VS = very slight hemolysis, and 0 = no hemolysis.

It is to be noted that although the amounts of amboceptor, complement, and cells, and the dilutions are the same in corresponding tubes of the different series, the results differ considerably. *B* and *C* are methods of activation which would hardly be used in practical work. They serve, however, to illustrate the extreme variation in results which may be obtained. It is evident in *B* that practically the entire amount of amboceptor was taken up by the first few corpuscles added, consequently they were the only ones to hemolyze, and there is little difference in the amount of hemolysis resulting between the highest and lowest tubes. *C*, on the other hand, as might be expected, approaches closely to an even sensitization. *A* and *D* are methods which are often used in complement titration, as for the Wassermann reaction, *A* probably more commonly than *D*. When a cell suspension is added to small tubes containing amboceptor, as was done in *A*, it may be noted that frequently a few cells will be distributed through the liquid, the greater bulk, however, remaining on the surface, along the side of the tube, or sinking to the bottom, thus affording an opportunity for a few cells to absorb more than their share of the amboceptor, the amount depending of course on the interval elapsing between the introduction of the cells and the shaking of the tube.

It is evident that to cause this inequality the absorption of amboceptor by corpuscles must be very rapid. The following experiment was carried out to obtain some idea of the amount of amboceptor absorbed by corpuscles in a given time.

Experiment II.—Amboceptor absorption in *A* with a 5 per cent. suspension of sheep corpuscles and corresponding immune rabbit serum; in *B*, with a 2 per cent. suspension of sheep corpuscles and corresponding immune rabbit serum. The amboceptor was so diluted as to contain 1, 2, and 5 units of amboceptor for equal quantities of the 5 per cent. suspensions of corpuscles.

(*a*) To 4 c.c. of diluted amboceptor in a centrifuge tube were added 4 c.c. of corpuscle suspension and the mixture was immediately centrifugalized at a speed of about 2,000 revolutions.

(*b*) Like *a*, except that it was centrifugalized after an interval of 2 minutes.

(*c*) Like *a*, except that it was centrifugalized after an interval of 5 minutes.

From each tube was taken 0.5, 1, 1.5, and 2 c.c. of the diluted amboceptor, 0.5 being approximately equivalent to 0.25 of the original amboceptor dilution, and to each of these amounts was added 0.2 c.c. of a 1:10 dilution of guinea pig serum (1 unit by previous titration) and 0.25 c.c. of corpuscle suspension, each tube being immediately shaken after the addition of the corpuscles. For the control tubes, the amboceptor was diluted with equal parts of salt solution.

After incubation at 37° C. for one hour the results were as follows:

Amboceptor.	0.5	1.0	1.5	2.0
(*A*) 5 per cent. suspension of sheep corpuscles				
1 unit of amboceptor				
Control	C.	C.	C.	C.
(*a*)	0	0	0	VS.
(*b*)	0	0	0	0
(*c*)	0	0	0	0
2 units of amboceptor				
(*a*)	VS.	P.	P.	AC.
(*b*)	0	VS.	P.	AC.
(*c*)	0	VS.	VS.	S.
5 units of amboceptor				
(*a*)	C.	C.	C.	C.
(*b*)	P.	C.	C.	C.
(*c*)	S.	P.	AC.	AC.
(*B*) 2 per cent. suspension of sheep corpuscles				
1 unit of amboceptor				
Control	C.	C.	C.	C.
(*a*)	0	S.	P.	AC.
(*b*)	0	0	0	VS.
(*c*)	0	0	0	0
2 units of amboceptor				
(*a*)	P.	C.	C.	C.
(*b*)	0	P.	AC.	AC.
(*c*)	0	VS.	S.	P.
5 units of amboceptor				
(*a*)	C.	C.	C.	C.
(*b*)	C.	C.	C.	C.
(*c*)	P.	C.	C.	C.

It appears from the above experiment that a 5 per cent. suspension of corpuscles almost completely absorbed one unit of amboceptor in the brief time necessary to centrifugalize off the cells. About three quarters of the two units of amboceptor appear to have been absorbed in the same length of time, while of the five unit amboceptor, one unit at least remained. Absorption by the 2 per cent. suspension was apparently not so rapid. About three quarters of the one unit, and about one half of the two unit amboceptor were absorbed in the time taken to centrifugalize, while of the five units, at least one unit remained even after absorption for two minutes, plus the time taken to centrifugalize.

Definite conclusions, however, as to the exact amount of amboceptor absorbed, or the relative speed of absorption by the 5 per cent. and 2 per cent. suspension of corpuscles, can not be drawn from such an experiment, for it is not known to what extent the failure of hemolysis may be due to inhibitory bodies produced by the addition of corpuscles for the purpose of amboceptor absorption (Sachs-Friedberger phenomenon). Such inhibitory bodies probably do not greatly influence the results obtained in this experiment, but if present it is natural to suppose that they are in larger amount after absorption with a 5 per cent. suspension of corpuscles, than after absorption with a 2 per cent. suspension.

Although the cells were centrifugalized from the fluid as quickly as possible, an interval of between one and two minutes probably elapsed before their complete removal. The following experiment was done to show that an appreciable amount of amboceptor is absorbed in a considerably shorter time than this.

Experiment III.—A 1:10 dilution of guinea pig serum was used in the amounts given below with sufficient salt solution in each tube to make the final total 1.25 c.c. A 5 per cent. and a 2 per cent. suspension of sheep corpuscles, and a 5 per cent. and a 2 per cent. suspension of calf corpuscles were used with one unit of the respective amboceptor. 0.5 c.c. of the corpuscle suspension activated as follows was added in series:

(*A*) 3 c.c. of the corpuscle suspension was poured into 3 c.c. of amboceptor dilution and quickly shaken.

(*B*) 0.5 c.c. of the corpuscle suspension was poured into 3 c.c. of amboceptor dilution, shaken, and 15 seconds later 2.5 c.c. of the corpuscle suspension were added.

(*C*) Like *B*, except that there was an interval of 30 seconds.

(*D*) Like B, except that there was an interval of 1 minute.

(*E*) Like *B*, except that there was an interval of 2 minutes.
After incubation at 37° C. for one hour, the results were as follows:

Guinea pig serum, dilution 1:10.	0.5	0.4	0.3	0.25	0.2	0.125	0.062	0.031	0.015
5 per cent. suspension of sheep corpuscles									
(*A*)	C.	C.	C.	C.	C.	C.	AC.	S.	VS.
(*B*)	C.	C.	C.	C.	AC.	AC.	P.	S.	0
(*C*)	C.	C.	C.	AC.	AC.	P.	S.	VS.	0
(*D*)	AC.	AC.	AC.	P.	P.	P.	S.	VS.	0
2 per cent. suspension of sheep corpuscles									
(*A*)	C.	C.	C.	C.	C.	AC.	VS.	0	0
(*B*)	C.	C.	C.	C.	C.	P.	VS.	0	0
(*C*)	C.	C.	C.	C.	AC.	P.	VS.	0	0
(*D*)	C.	C.	AC.	AC.	P.	S.	VS.	0	0
(*E*)	AC.	P.	P.	S.	S.	VS.	VS.	0	0
5 per cent. suspension of calf corpuscles									
(*A*)	C.	C.	C.	C.	C.	C.	AC.	VS.	0
(*B*)	C.	C.	C.	C.	C.	AC.	P.	VS.	0
(*C*)	C.	C.	AC.	AC.	AC.	AC.	P.	VS.	0
(*D*)	C.	C.	AC.	AC.	AC.	P.	VS.	VS.	0
(*E*)	AC.	AC.	P.	P.	S.	S.	VS.	VS.	0
2 per cent. suspension of calf corpuscles									
(*A*)	C.	C.	C.	C.	AC.	P.	VS.	VS.	0
(*B*)	C.	C.	C.	C.	AC.	P.	VS.	0	0
(*C*)	C.	C.	C.	AC.	P.	VS.	VS.	0	0
(*D*)	C.	C.	AC.	P.	VS.	VS.	VS.	0	0
(*E*)	AC.	AC.	AC.	P.	VS.	VS.	VS.	0	0

It will be seen by the foregoing experiment that a considerable effect is produced on the degree of hemolysis obtained by a contact of even fifteen seconds of a portion of the corpuscles with the amboceptor before the introduction of the remainder. The effect is somewhat less marked with the 2 per cent. than with the 5 per cent. suspension. This, we believe, is what takes place in greater or less degree in any serum titration when an even distribution of the corpuscles throughout the amboceptor dilution is not at once obtained.

It is probably impossible to obtain an absolutely even sensitization. The nearest approach to this is produced by quickly pouring the diluted immune serum into an equal volume of the corpuscle suspension, which is shaken during and for a short period after the mixing (experiment I, *D*). If the corpuscles are poured slowly into the amboceptor or introduced in separate lots, as with a five or ten cubic centimeter pipette, unequal sensitization will result. This is

illustrated in an extreme degree by the method of sensitization used in experiment I, *B*, that of dropping the cells into the amboceptor. It is to be noted that the reading is not only higher with an even sensitization, but is also sharper, that is, the change from complete hemolysis to entire lack of hemolysis, instead of being gradual is quite sudden (experiment I, *A* and *D*).

The importance in practical work of obtaining as uniform a distribution of amboceptor as possible is obvious, as well as the necessity in any comparative titration of using suspensions of activated cells in the various titrations which are exactly alike as to the distribution of amboceptor. The importance of complement titration for the Wassermann or other complement absorption tests is rightly emphasized. If, however, the same method of sensitization is not used in this titration as is used in the final stage of the Wassermann reaction, the titration is of little value as an index of the activity of the serum. It is a common practice to introduce amboceptor and corpuscles separately in a complement titration (experiment I, *A*) while corpuscles previously sensitized in bulk are used in the Wassermann reaction. By the latter method it is much easier to approximate an even sensitization (experiment I, *D*). Thus the reading obtained in a complement titration in which amboceptor and corpuscles are added separately would lead one to use an excess of complement in performing the reaction.

In an amboceptor titration, the immune rabbit serum and corpuscles are usually introduced separately (to employ separate lots of corpuscles previously activated with each dilution would be an exceedingly tedious task), yet if one accepts the highest dilution with which complete hemolysis is obtained as the titre of the serum, and with this dilution titrates the same complement as was used in the amboceptor titration, hemolysis will be obtained, provided more evenly sensitized corpuscles are here used, with considerably less complement than was used in the amboceptor titration.

This may be illustrated by reference to experiment III. It is stated in this experiment that one unit of amboceptor was used. Both the anti-sheep and the anti-calf amboceptor were titrated before this experiment with 0.25 of a cubic centimeter of the same dilution of guinea pig serum as was used in the experiment. The anti-sheep serum was found to give complete hemolysis of the 5 per

cent. suspension of corpuscles in a dilution of 1 to 800, and incomplete hemolysis in 1 to 1,000; the anti-calf gave complete hemolysis of the 5 per cent. suspension of corpuscles in a dilution of 1 to 400, and incomplete hemolysis in 1 to 600. In the experiment, however, in which they were used in dilutions of 1 to 800 and 1 to 400, respectively, we find that when previously sensitized corpuscles were used (experiment III, *A*), both gave complete hemolysis with one half the amount of complement (0.125 of a cubic centimeter) with which they were titrated. A retitration of the anti-sheep serum with 0.25 of a cubic centimeter of the complement dilution, 0.5 of a cubic centimeter of the corpuscle suspension previously activated being employed for each dilution by rapidly mixing 5 cubic centimeters of a 5 per cent. suspension of corpuscles with an equal amount of the proper amboceptor dilution, gave complete hemolysis in a dilution of 1 to 1,200. We must conclude, then, that an amboceptor as well as a complement titration is influenced by the evenness of the sensitization of the corpuscles.

CONCLUSIONS.

The absorption of amboceptor by corpuscles is rapid, a considerable amount being absorbed in as short a period as fifteen seconds. In the sensitization of corpuscles, the amount of amboceptor absorbed by the different corpuscles is not uniform, the inequality depending on the time taken in obtaining an even distribution of the corpuscles through the diluted immune serum. Amboceptor absorption is apparently influenced by the concentration of the corpuscles, being more rapid with a 5 per cent. than with a 2 per cent. suspension, and thus the stronger concentration is more susceptible to inequality of sensitization from variations in the method of activation.

In experimental work, it is of importance to obtain as equal a sensitization as possible. It is essential that in comparative titrations the same method of activation be employed in the several titrations, and where possible it is advisable that all the cells used be sensitized together in bulk.

The results obtained in titrating hemolytic sera and complement depend to a considerable extent upon the evenness of sensitization of the corpuscles.

ON THE PRESENCE OF NUCLEASE IN CARCINOMA.*

By EDWARD H. GOODMAN, M.D.

(From the Laboratory of Dr. John H. Musser, Philadelphia, Pa.)

In spite of the voluminous literature that has been published on carcinoma, and the many researches devoted to this question, it is surprising to find how few investigators are studying the biochemistry of the cancer cell itself, or rather how few articles have appeared on this subject. The morbid anatomy and the microscopic pathology of the carcinoma have been assiduously and thoroughly studied, and researches along the less well known lines of parasitology and protozoölogy have been conducted with an ardor that is deserving of better results than have been apparent. Of late years, these well worn paths have been somewhat deserted, and attention has been directed to that part of the subject which has to do with the chemical activities of the living cancer cell.

That the cancer cell behaves differently from any other cell of the normal organism there can now be no doubt. Petry (1) found that there was a great increase in nucleoprotein in carcinoma, which he ascribed to the richness of the gland in nuclei, and in a later paper (2) he was able to show that autolytic changes take place by means of which diamino acids, hypoxanthin, lysin, tyrosin, leucin, and xanthin were produced. Beebe (3) demonstrated the presence of glycocoll, leucin, tyrosin, tryptophan, and in addition, found pentose in large amounts, also a peculiar carbohydrate which stained blue with iodin, but which, like glycocoll, was readily soluble in water. When hydrolyzed, it stained red with iodin and reduced copper. Neuberg and Milchner (4) have corroborated the high percentage of pentose in carcinoma. Wolff (5) found in cancer that there was more albumin than globulin, while in the majority of normal organs the amounts were about equal.

Bergell and Dörpinghaus (6) discovered that diamino acids com-

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prised about one third of the total nitrogen, and that alanin, phenylalanin, glutaminic acid, and aspartic acid were also present in large amounts (5 to 10 per cent.). Most striking was the low proportion of leucin (5 to 6 per cent.), whereas in the normal cell, as high as 20 per cent. is found.

Blumenthal and Wolff (7) suggested that autolytic changes in carcinoma might be due to a different composition of the protein which renders it more vulnerable to attack, and not necessarily to the presence of increased amounts of heterolytic ferments. In confirmation of this theory, they showed that cancerous material was quite resistant to pepsin and was easily broken up by trypsin. In direct contrast is the reaction of normal tissue, which is readily digested by pepsin and not by trypsin. Confirmation of this behavior of cancer toward ferments is given by Neuberg (8).

That carcinoma can break down not only its own tissue, but can also attack tissue quite foreign to it has been proven by Neuberg (9) who showed that autolysis of the lung is increased by the addition of cancer juice. This heterolytic ferment, to use an expression coined by Jacoby, has never been found in normal organs, and Neuberg queries whether the cause of cachexia is to be looked for in this ferment. A polypeptid-splitting ferment has been found in cancer by Neubauer and Fischer (10), and Neuberg (11) states that tumor juice can break up d-alanyl-glycylglycin to glycocoll and d-alanyl-glycin, while normally it is broken up to d-alanin and glycylglycin.

It is at once apparent that the work briefly cited above has a very important bearing on the cancer question, inasmuch as the presence of a ferment or ferments which are able to attack albumin in such a way must be regarded as a grave menace. Not only may such a ferment be a menace locally, by its slowly progressing invasion of the contiguous normal tissues, but it is not fanciful to suppose that under certain conditions it may enter the blood stream, and through this medium exercise its destructive function on the organs as a whole. This destructive function of an heterolytic ferment circulating unrestrained through body tissues may be manifested in the rapid and extreme loss of weight noted in carcinoma patients and in their little understood cachexia.

Since the vital part of any living cell is its nucleus, successful attack directed toward this not impregnable fortress would, after its annihilation, be followed by rapid destruction of the remainder of the cell. The nucleus of a cell is composed for the most part of nucleic acid, which, under certain conditions may be readily broken down by a specific ferment, in this case nuclease. The task which we set for ourselves was to ascertain whether a nuclease could be found in carcinomatous tissue, and if so, what bearing its presence might have on the cancer question.

Material.—Non-ulcerating mammary carcinomata of women were chosen for this work; non-ulcerating in order to avoid bacterial contamination, and from the breast on account of the comparative ease with which neoplasms of this organ can be separated from the glandular tissue. As soon as the material was procured at operation it was placed in the ice chest, and within twenty-four hours work was begun with it.

The tumor was carefully separated from the breast tissue, chopped into small pieces, and ground to a fine pulp in a meat chopper. The substance was then put in a mortar, thoroughly ground with sand and normal salt solution, and the juice was finally expressed in a Buchner press. The resulting cancer juice was measured and made up to a definite amount with salt solution and finally divided into four equal portions.

Preparation of the Sodium Salt of Nucleic Acid.—The method of Neumann (12) was at first followed exactly, and the yield of the salt was most unsatisfactory. Through the kindness of Professor Schittenhelm, the writer was given the benefit of some valuable modifications in technique, by following which there resulted the easy recovery of large amounts of the acid. The method which proved most satisfactory follows.

One kilogram of fresh thymus gland is boiled in a weak acetic acid solution, which renders the removal of connective tissue, fat, and blood-vessels a matter of no great difficulty. This preparation of the glands, however, should be carried out with great scrupulousness. After the glands are as free as possible from foreign material, they are ground up in a meat chopper, and the pulp is put in two liters of boiling water containing 100 cubic centimeters

of a 33 per cent. solution of sodium hydroxid and 200 grams of sodium acetate.

In Neumann's paper, it is recommended to boil the solution on a water bath for one half hour, but, in accordance with Professor Schittenhelm's suggestion, a large porcelain dish was used. The above solution was brought to boiling in this dish and the gland pulp was put in and thoroughly stirred. After all the material has gone into solution, the boiling is continued for fifteen minutes and the mixture neutralized with 50 per cent. acetic acid.

This part of the technique must be conducted with extreme caution, and the solution when properly neutralized must change blue litmus red and red litmus blue. It is far better to have the solution faintly alkaline than faintly acid, for in the latter event the subsequent filtering will be impossible. When exactly neutralized, the material is filtered through a folded filter in a hot water funnel. Everything must be kept hot, otherwise the fluid gelatinizes and cannot be filtered off.

The clear, or, at most, slightly cloudy, amber colored filtrate is evaporated on a water bath to about 500 cubic centimeters, cooled to 50° C., and then precipitated with an equal volume of 95 per cent. alcohol; the sodium nucleate settles in a coarse precipitate. The supernatant alcohol is decanted, the precipitate is repeatedly washed with 95 per cent. alcohol, then with absolute alcohol, and finally with repeated changes of ether. It is sucked off, washed again with alcohol, then with ether, and is finally dried in a desiccator or on heavy filter paper. From 1,000 grams of thymus the yield of the sodium salt of nucleic acid by this method is about thirty to thirty-five grams.

Tests with Cancer Juice.—Four grams of the sodium salt were dissolved in 100 cubic centimeters of hot water and allowed to cool. One portion of cancer juice was put in a flask containing 100 cubic centimeters of a 4 per cent. solution of sodium nucleate (flask 1); one portion was placed in a flask by itself (flask 2); one portion was boiled and put in a flask with a 4 per cent. solution of sodium nucleate (flask 3); and one portion was boiled and put in a flask by itself (flask 4).

After a period of time that varied in each series, the flasks

were removed from the incubator, a few cubic centimeters of concentrated sulphuric acid were added to destroy any free nuclein bases, and the mixture was filtered. To the filtrate was added mercuric sulphate solution (prepared by dissolving seventy-five grams of mercuric oxid in 500 cubic centimeters of a hot 15 per cent. solution of sulphuric acid), the precipitate was filtered off, the residue was suspended in water to which hydrochloric acid had been added, and the sulphurated hydrogen was passed through the whole. The resulting precipitate was filtered off, the filtrate was freed from sulphurated hydrogen by air, and was precipitated with ammoniacal silver solution (prepared by dissolving twenty-six grams of silver nitrate in water, adding ammonia until the precipitate of brown silver oxid goes again into solution, and then water making the solution equal to 1,000 cubic centimeters). The precipitate was filtered off, well washed, suspended in water, warmed, and while warm was broken up with hydrochloric acid. The silver chlorid was filtered off, and a small quantity of sulphurated hydrogen was conducted through the filtrate, which was again filtered. The filtrate was then evaporated to dryness, the residue was taken up in dilute hydrochloric acid, filtered, and evaporated to dryness in a weighed platinum dish.

Test for Free Nuclein Bases.—After the dried filtrate from the various flasks was weighed, the test for free nuclein bases, as described by Burian (13) and modified by Pauly (14), was made as follows: Two grams of finely powdered sulphanilic acid are shaken with three cubic centimeters of water and two cubic centimeters of hydrochloric acid (concentrated), and in small portions within a minute's time a solution of one gram of potassium nitrite in one cubic centimeter of water is added, the solution being heated after each addition. The precipitate of diazobenzolsulphonic acid is sucked off after a few minutes, and washed with a small amount of water. To the solution to be tested is added an excess of soda solution and three to five cubic centimeters of a freshly made solution of the diazobenzolsulphonic acid. If free bases are present, there will appear within three minutes a dark cherry red color which becomes orange on the addition of an acid.

EXPERIMENT 1.

In Incubator for 27 Days.

		Purin bases.
Flask 1.	50 c.c. cancer juice with 100 c.c. 4 per cent. sodium nucleate....	0.4650
Flask 2.	50 c.c. cancer juice	0.1020
Flask 3.	50 c.c. cancer juice (boiled) with 100 c.c. 4 per cent. sodium nucleate	0.0446
Flask 4.	50 c.c. cancer juice (boiled). Not estimated.	

EXPERIMENT 2.

In Incubator for 4 Days.

No. 1.	60 c.c. cancer juice with 100 c.c. 4 per cent. sodium nucleate....	0.5004
No. 2.	60 c.c. cancer juice	0.1301
No. 3.	60 c.c. cancer juice (boiled)	0.0878

EXPERIMENT 3.

In Incubator for 3 Days.

No. 1.	50 c.c. cancer juice with 100 c.c. 4 per cent. sodium nucleate.....	0.2471
No. 2.	50 c.c. cancer juice	0.0884
No. 3.	50 c.c. cancer juice (boiled).....	0.0188

In all the tests given above, the presence of free nuclein bases was indicated.

The results of these three experiments offer proof of the presence of nuclease in carcinomata. In the flasks containing sodium nucleate and carcinoma juice, a greater amount of the purin bases was present than in those containing cancer juice that was allowed to autolyze without the addition of nucleic acid, and these flasks contained greater amounts of the purin bases than the flasks in which the ferments had been destroyed by boiling.

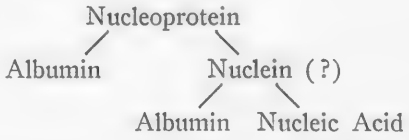
It must be inferred that while the cancer is still growing in the human organism, the nuclease of the cancer is active. This is indicated by the presence of the purin bases in the flasks that contained boiled cancer juice, for the action of the ferment in the cancer was inhibited by cold immediately after the removal of the tumor from the body, and the ferment was later destroyed by heat. The purin bases in these flasks must, therefore, have been produced while the tumor was growing on its host.

The nuclease continues its activities in the incubator. This is shown by the contents of the No. 2 flasks, for the increase in the

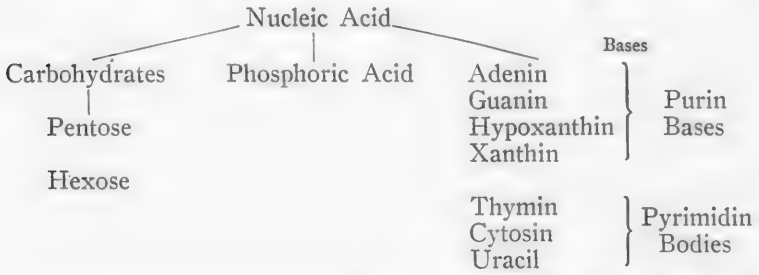
purin bases in these must represent the destruction of the nuclei of the carcinoma cells by the ferment (autolysis).

In the No. 1 flasks, the quantity of purin bases far exceeded the endogenous purins (No. 3 flasks), and the purins derived by autolysis (No. 2 flasks), and must have come from the nucleic acid which had been broken down by the nuclease inherent in the cancer.

A diagrammatic representation of the method of cleavage of nucleoprotein is here reproduced.



When the nucleic acid is attacked, it is probably split as follows:



Proof of the carbohydrate split product has been offered by Beebe (3) and Neuberg and Milchner (4).

Entrance into the blood-stream of a ferment that is able to break down the vital component of a living cell would well explain the rapid destruction of the protein tissue of the organism, and might have some bearing on the appearance of cachexia. Evidences of increased nuclein metabolism are offered by the increase of purin bases in the urine of cancer patients (Brandenburg (15) and Blumenthal (16), and by the increase in the amount of phosphates (Lewin (17), von Noorden (18)), although the former is by no means a constant finding (Setti (19)). Phosphates have also been found in the stomach washing of cases of gastric carcinoma (Goodman (20)).

Having found a nuclease in carcinomatous tissue, we intend to search for it in the blood and urine of patients suffering from cancer, and in the gastric secretion of cases of gastric neoplasms. Should nuclease be at all concerned in the malignancy of carcinoma, injection of it into tumors should hasten their growth, and perhaps its injection into lower animals might reveal phenomena bearing on the cancer question.

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IMMUNOCHEMICAL STUDIES WITH PEPTONES.*

I. General Studies with Peptones and the Immune Hemolytic System.[1]

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Ever since the classic experiments of Schmidt-Mülheim (1) and Fano (2) the peptone bodies arising from peptic digestion have been known to possess toxic properties. Hofmeister (3) has shown that when peptones are brought directly into the circulation of an animal, only a small portion appears in the urine, and that blood withdrawn shortly after injecting the peptones fails to show their presence when tested by the usual chemical means. The difficulty of identifying such bodies in mixtures with other proteins (such as are present in the blood) arises from the crude methods at our disposal.

From a physicochemical point of view, the albumoses and peptones behave very much like the other complex protein substances, differing from the latter only in respect to coagulation by heat and diffusibility through semipermeable membranes. Rossi (8), however, draws attention to the fact that in the separation of peptones from organs or blood by means of coagulation or precipitation with salts much of the peptone is lost. In other words, the native proteins in the course of coagulation or salting out carry part of the peptones out of solution. This is confirmed by the experiments of Abderhalden and Oppenheimer (4), who have recently shown that the

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[1] For purposes of convenience, the present work will be reported in several parts:

I. General studies with peptones and the immune hemolytic system.

II. The effect of lecithin on the hemolytic action of certain peptones. (*a*) The activating action of lecithin upon peptone hemolysis, and (*b*) The resemblance of the hemolytic peptones to cobralysin.

III. The action of deuteroalbumose B on solanin hemolysis.

IV. The cobralysin activator of horse serum.

separation of the albumoses and peptones from the serum or blood proteins, especially when the former are present in small quantities, is practically impossible.

There is no longer any doubt that substances resembling the albumoses and peptones can arise *intra vitam* as the result of autolytic or cytolytic processes, and can in themselves be responsible for a number of pathological states.

Whether peptones enter the circulation from the intestinal tract unchanged, is still an unsettled question. A method which would enable us to determine the presence of the peptones in the blood would, therefore, be of the utmost importance in the study of the physiological and pathological significance of these substances. As stated above, chemistry alone does not offer such a means.

In immunological work, certain changes in the reactions of blood sera are known to occur at the height of digestion which are attributed to the entrance of products of digestion into the circulation. Hewlett (9) has succeeded in showing that marked changes in the immune reactions of blood plasma take place after intravenous injections of Witte's peptones. Löwenstein (5) has demonstrated that *in vitro* peptones can absorb complement, and in that way can interfere with immune reactions.

The object of the present investigation is, first, to ascertain the effect of different albumose and peptone bodies upon certain immunochemical phenomena (chiefly those of hemolysis), and, second, to establish, if possible, from the observations made a method for determining these bodies in mixtures with the proteins of the blood.

In the experiments made, two types of proteoses were used; namely, Witte's peptone, and a peptone mixture resulting from the artificial digestion of casein with pepsin and hydrochloric acid. The individual peptone bodies were separated and refined, according to the method of Pick.

As is known, Witte's peptone is a mixture of albumoses and peptones produced by the digestion of blood fibrin. The object of using the casein peptones was to ascertain whether or not corresponding peptones from different sources, or native proteins, would give similar immunochemical reactions. Of the proteoses present in Witte's peptone, the following were especially studied:

I. Primary albumoses { protalbumose. / heteroalbumose.

II. Secondary albumoses { deuteroalbumose A. / deuteroalbumose B.

III. Peptones { peptone A. / peptone B.

With the exception of heteroalbumose, which casein does not yield on peptic digestion, corresponding proteoses of casein were used in the tests.

THE EFFECT OF DIFFERENT PEPTONES ON RED BLOOD CORPUSCLES.

In the experiments made, the individual peptone fractions, after proper separation and dialysis, as well as the original peptone mixtures, were dissolved in normal saline solution and the solutions were rendered neutral to litmus. Starting with a 10 per cent. solution of each of these substances, different dilutions were made and tested, first of all upon washed red blood cells of the sheep.

The results obtained indicate that the action of the different bodies varies; some show a distinct hemolysis, others do not, and still others cause an agglutination of the cells with or without a reduction of color. Grouping the peptones, therefore, according to the above reactions for sheep red blood cells we have:

1. Those that hemolyze
   - peptone mixtures { Witte / casein
   - deuteroalbumose B { Witte / casein
   - peptone B { Witte / casein

2. Those that do not hemolyze
   - protalbumose { Witte / casein
   - heteroalbumose { Witte
   - deuteroalbumose A { Witte / casein
   - peptone A { Witte / casein

3. Those that agglutinate
   - peptone B { Witte / casein

The hemolysis caused by deuteroalbumose B is quite distinct even in high dilutions, and is more marked with the deuteroalbumose B from casein than from Witte's peptone.

In view of the results obtained with sheep red blood cells, it seemed advisable to test the action of the proteoses upon other species of red blood cells. The washed corpuscles of thirteen different species of blood were tested against the peptones. The results obtained are summarized in table I.

The effect produced by proteoses upon different species of red blood cells manifests itself, as previously stated, in three phases; namely, hemolysis, reduction of color, and agglutination.

Some of the peptone fractions, namely, heteroalbumose (Witte), deuteroalbumose A (Witte and casein), protalbumose (casein), peptone A (casein), are absolutely inactive towards all the species of red blood cells. Agglutination of human corpuscles occurs with the whole peptone mixture (Witte). Color reduction is produced upon a number of cell species by deuteroalbumose B (Witte), peptone A (Witte), peptone B (Witte), deuteroalbumose B (casein), and peptone B (casein). Most of these fractions are capable of producing an agglutination of the cells before hemolysis, or precipitation after hemolysis; usually precipitation (or agglutination) of the cells occurs before hemolysis. With the peptone B, both from the casein and Witte mixtures, the power to precipitate is greater than that to hemolyze, and, with the stronger concentrations of these two peptones, the precipitation is either exhibited first, or else there occurs no hemolysis at all. In the latter case, the precipitating action is overcome by dilution of the peptones, whereupon the hemolytic action becomes evident.

That the cells which are agglutinated or precipitated prior to being hemolyzed remain intact, can be demonstrated with the microscope. The effect upon the cells may be one of fixation, such as is produced by other substances, because different hemolytic agents and even distilled water fail to dissolve them. The precipitating effect of the above peptone fractions is demonstrable in tests made with laked blood.

The peptone fractions hemolyze the different species of red blood cells in the following order:

TABLE I.

Species of red blood cells.	Peptone mixture (Witte).	Protalbumose (Witte).	Heteroalbumose (Witte).	Deuteroalbumose A (Witte).	Deuteroalbumose B (Witte).	Peptone A (Witte).	Peptone B (Witte).	Protalbumose (casein).	Deuteroalbumose A (casein).	Deuteroalbumose B (casein).	Peptone A (casein).	Peptone B (casein).
Human	0	0	0	0	1:20++ 1:40=0	1:10 +++	1:40 +++	0	0	1:10++ 1:20+	0	1:20 +++
Beef	0	0	0	0	0	1:10 +++	1:10=0 1:20++	0	0	0	0	1:10=0 1:20++
Horse	0	1:10[2]++[3] 1:20+	0	0	1:10+++ 1:20++	1:20 +++	1:40+++ 1:80+++	0	0	1:20 +++	0	1:10=0 1:40+++
Goat	0	1:20 +++	0	0	1:20 ++	1:20 +++	1:80 +++	0	0	1:40 +++	0	1:10=0 1:40+++
Sheep	1:10 +	0	0	0	1:10+++ 1:20++	0	1:10=0 1:20+++ 1:40+++	0	0	1:20+++ 1:40+	0	1:10=0 1:40+++
Rabbit	0	0	0	0	0	0	1:20 +++	0	0	0	0	1:20 +++
Cat	0	0	0	0	1:10+++ 1:20++ 1:40+	1:10 ++	1:20=0 1:40+++	0	0	1:20 +++	0	1:10=0 1:20+++ 1:40+++
Dog	0	0	0	0	1:10++ 1:20+	1:10+++ 1:20++	1:10=0 1:20+++ 1:40++	0	0	1:20++ 1:40+	0	1:10=0 1:20+++ 1:40++
Mouse	0	1:20+++ 1:40++	0	0	1:20+++	1:10+++ 1:20++	1:40 +++	0	0	1:40 +++	0	1:10 +++
Rat	0	0	0	0	0	0	1:20 +++	0	0	0	0	1:20 +
Goose	0	0	0	0	0	0	0	0	0	0	0	0
Hen	0	0	0	0	0	1:10 +	1:10 +	0	0	0	0	1:10=0 1:20+
Pig	0	0	0	0	0	1:10+	1:10+ 1:20++	0	0	0	0	1:10=0 1:20++ 1:40+

[2] Peptone mixture (Witte) causes agglutination of human red blood corpuscles up to dilutions of 1:40.

[3] The numerals represent the dilutions of the peptones and the sign + represents the degree of hemolysis.

Peptone B (Witte and casein)	hemolyzes 12 kinds of red blood cells.
Peptone A (Witte)	hemolyzes 10 kinds of red blood cells.
Deuteroalbumose B (Witte)	hemolyzes 8 kinds of red blood cells.
Deuteroalbumose B (casein)	hemolyzes 7 kinds of red blood cells.
Protalbumose (Witte)	hemolyzes 3 kinds of red blood cells.
Peptone mixture (Witte)	hemolyzes 1 kind of red blood cells.

The results so far indicate that the peptone bodies possess certain distinct properties in respect to lysis and agglutination of red blood cells. Corresponding fractions exhibit like functions. The different members of the same mixture (Witte or casein), though crudely separated from each other in a chemical sense, show different properties. The heteroalbumose and deuteroalbumose A (Witte), as well as protalbumose and deuteroalbumose A (casein), and peptone A (casein), though closely placed, in the order of their chemical grouping between bodies that are active in respect to lysis and agglutination, are themselves quite inactive.

As in other tests for hemolysis, the action of the peptone depends upon two factors: (1) their own hemolytic action; and (2) sensibility or sensitiveness of the red blood cells. The sensitiveness of the different red blood cells for the hemolytic action of peptone fractions is as follows:

Red blood cells of the horse, goat, and dog	are sensitive to 6 peptones.
Red blood cells of man, sheep, and cat	are sensitive to 5 peptones.
Red blood cells of beef, pig, and hen	are sensitive to 3 peptones.
Red blood cells of the rabbit and mouse	are sensitive to 2 peptones.
Red blood cells of the rat	are sensitive to 1 peptone.
Red blood cells of the goose	are sensitive to 0 peptone.

In a general way, as far as the direct action upon red blood cells is concerned, there are more inactive members present among the peptone fractions than active ones, and the negative conduct of the peptone mixtures indicates that the inhibitory action of these bodies predominates over the others. Having thus inquired into the action of the peptone fractions upon red blood cells themselves, further tests were made upon the peptones with respect to their effect on the different immune elements of serum.

THE EFFECT OF THE DIFFERENT PEPTONES ON COMPLEMENT.

As previously stated, Löwenstein (4) has already shown that the peptones, like many other substances, interfere with the complement

of normal and immune blood sera. In the following experiment, an attempt is made to establish a qualitative and quantitative rela-

TABLE II A.

Complement.		100 per cent.	50 per cent.	25 per cent.	10 per cent.	5 per cent.	Saline. Red blood cell control.
Peptone mixture (Witte)	10 per cent.	+++[4]	0	0	0	0	0
	5 per cent.	+++	+++	+++	+?	0	0
	2.5 per cent.	+++	+++	+++	+++	+++	0
	1.25 per cent.	+++	+++	+++	+++	+++	0
Control (amboceptor)	Sodium chlorid	+++	+++	+++	+++	+++	0

Each tube contained:
- 0.2 c.c. of peptone solution.
- 0.2 c.c. of complement solution.
- 0.2 c.c. of amboceptor, 1 : 1,000 dilution.
- 0.2 c.c. of 5 per cent. sheep red blood cell suspension.
- 0.2 c.c. of saline solution.

TABLE II B.

	Per cent.	Complement 100 per cent.	Complement 10 per cent.
1. Peptone mixture (Witte)	10	+++[5]	0
2. Peptone mixture (Witte)	1	+++	+++
3. Protalbumose (Witte)	10	+++	+++
4. Protalbumose (Witte)	1	+++	+++
5. Heteroalbumose (Witte)	10	+++	+++
6. Heteroalbumose (Witte)	1	+++	+++
7. Deuteroalbumose A (Witte)	10	0	0
8. Deuteroalbumose A (Witte)	1	+++	++
9. Deuteroalbumose B (Witte)	10	+++	0
10. Deuteroalbumose B (Witte)	1	+++	+++
11. Peptone A (Witte)	10	+++	0
12. Peptone A (Witte)	1	+++	+++
13. Peptone B (Witte)	10	+	0
14. Peptone B (Witte)	1	+++	+++
15. Protalbumose (casein)	10	+++	+?
16. Protalbumose (casein)	1	+++	++
17. Deuteroalbumose A (casein)	10	+++	+?
18. Deuteroalbumose A (casein)	1	+++	+++
19. Deuteroalbumose B (casein)	10	+++	0
20. Deuteroalbumose B (casein)	1	+++	+++
21. Peptone A (casein)	10	+++	+++
22. Peptone A (casein)	1	+++	+++
23. Peptone B (casein)	10	+	0
24. Peptone B (casein)	1	+++	+++

Each tube contained:
- 0.2 c.c. of peptone solution.
- 0.2 c.c. of hemolysin, 1 : 200 dilution.
- 0.2 c.c. of complement solution.
- 0.2 c.c. of sheep red blood cell suspension.
- 0.2 c.c. of saline solution.

[4] The degree of hemolysis is indicated by +.
[5] The degree of hemolysis is indicated by +.

TABLE III.

Peptone Fractions.	Sensitization Experiments.													
	I.							II.				III.		
	Per cent.	*a*		*b*		*c*		*a*		*b*				
		Hemolysis.	Agglutination.	Hemolysis.	Agglutination.	Hemolysis.	Agglutination.	Hemolysis.	Agglutination.	Hemolysis.	Agglutination.	Hemolysis.	Agglutination.	
Peptone (Witte)	10	0	+	0	++	+++	0	+++		0	++	+++	0	
Peptone (Witte)	1	0	0	0	++	+++	0	+++		0	++	+++	0	
Protalbumose (Witte)	10	0	0	0	0	+++	0	+++		0	++	+	0	
Protalbumose (Witte)	1	0	0	0	++	+++	0	+++		0	++	++	0	
Heteroalbumose (Witte)	10	0	0	0	++	+++	0	+++		0	++	+++	0	
Heteroalbumose (Witte)	1	0	0	0	++	+	0	+++		0	++	++	0	
Deuteroalbumose A (Witte)	10	0	0	0	++	+	0	+++		0	++	+	0	
Deuteroalbumose A (Witte)	1	0	0	0	++	+++	0	+++		0	++	+++	0	
Deuteroalbumose B (Witte)	10	0	0	0	0	−	0	+++		0	+	++	0	
Deuteroalbumose B (Witte)	1	0	0	0	++	+	0	+++		0	++	+++	0	
Peptone A (Witte)	10	0	0	0	0	−	0	+++		0	++	0	0	
Peptone A (Witte)	1	0	0	0	0	+++	0	+++		0	++	+++	0	
Peptone B (Witte)	10	0	+++	0	++	0	++	+++		0	++	0	++	
Peptone B (Witte)	1	0	0	0	++	+++	0	+++		0	++	++	0	
Protalbumose (casein)	10	0	0	0	++	+++	0	+++		0	++	+++	0	
Protalbumose (casein)	1	0	0	0	++	+++	0	+++		0	++	+++	0	
Deuteroalbumose A (casein)	10	0	0	0	++	+	0	+++		0	++	++	0	
Deuteroalbumose A (casein)	1	0	0	0	0	+++	0	+++		0	++	+++	0	
Deuteroalbumose B (casein)	10	0	0	0	0	−	0	+++		0	0	0	0	
Deuteroalbumose B (casein)	1	0	0	0	++	++	0	+++		0	0	+++	0	
Peptone A (casein)	10	0	0	0	++	+++	0	+++		0	0	+++	0	
Peptone A (casein)	1	0	0	0	++	+++	0	+++		0	0	+++	++	
Peptone B (casein)	10	0	+++	0	++	0	++	+++		0	0	0	0	
Peptone B (casein)	1	0	++	0	++	+++	0	+++		0	0	+	0	

I. Sensitization with peptones + { (*a*) complement. (*b*) amboceptor, 1 : 1,000. (*c*) amboceptor + complement.

II. Sensitization with amboceptor + { (*a*) complement. (*b*) peptone fraction.

III. Sensitization with peptones + amboceptor, 1 : 500.

tionship between this labile element of serum, and the different peptone fractions.

This experiment shows the absorption of complement by Witte's peptone mixture and the quantitative relation between the two.

The results show that the absorption of complement is proportional to the amount of peptone present in the complement-serum-peptone mixture (tables II A and II B).

Low concentrations of peptone appear to be without effect on the complement. The complement of serum and the peptone together do not form a complete hemolytic system; that is to say, peptone can not act as amboceptor although it absorbs complement. When sheep red blood cells are first sensitized with immune amboceptor and then treated with the different peptone fractions, it is found, as will be shown later, that the latter can not take the place of complement; and that when guinea pig complement serum is added, hemolysis occurs in the presence of some of the peptones, but not in the presence of others (table III).

Briefly, the tests (table II B) show that on the addition of guinea pig complement to the sensitized blood cells in the presence of peptone fractions, hemolysis occurs under the following conditions:

1. When only 1 per cent. peptone mixture (Witte) is present.
2. When only 1 per cent. protalbumose (Witte) is present.
3. Heteroalbumose does not interfere with the complement in any concentration.
4. Deuteroalbumose B does not interfere with hemolysis in higher dilutions. In concentration it dissolves red blood cells by itself.
5. Peptone A does not interfere with complement action up to 10 per cent. concentration.
6. Peptone B does not interfere with hemolysis of sensitized red blood cells in 1 per cent. concentration, but it does interfere in higher concentrations.

On the other hand, hemolysis, that is, complement action, is interfered with by 10 per cent. peptone (Witte); 10 per cent. protalbumose (Witte); 1 per cent. deuteroalbumose A (Witte); 10 per cent. peptone B (Witte and casein); 10 per cent. protalbumose; 10 per cent. deuteroalbumose A; and 10 per cent. peptone B.

The foregoing shows that the peptones as a class can not be said to absorb complement. They show marked individual differences in this respect.

THE EFFECT OF PEPTONE UPON THE AMBOCEPTOR IN IMMUNE SERUM.

This element in the hemolytic system of immune serum is much more stable. From what is known, interference with hemolysis can arise from the intervention of substances capable of absorbing the complement, but not as readily by directly influencing the amboceptor. The effect of peptones upon amboceptor has been tested in two ways: first, by testing the efficiency of amboceptor action upon red blood cells in the presence of the different peptone bodies; secondly, by testing the capacity of the amboceptor for binding red blood cells after treatment (sensitization) of the latter with the peptone fractions.

The following experiment is intended to show the effects of mixtures of peptone fractions with immune amboceptor upon the hemolytic action of the amboceptor. In this experiment 0.5 of a cubic centimeter of hemolysin (1:5 dilution) and 0.5 of a cubic centimeter of peptone fractions (in 10 per cent. and 1 per cent. concentrations, respectively) were kept in the thermostat at 37° C. for one hour; then each mixture was diluted so as to give dilutions of hemolysin of 1:250, 1:500, 1:750, and 1:1,000, respectively. Each tube contained 0.2 of a cubic centimeter of the diluted hemolysin peptone mixture, 0.2 of a cubic centimeter of 5 per cent. suspension of sheep red blood cells, 0.2 of a cubic centimeter of 10 per cent. guinea pig complement, and 0.4 of a cubic centimeter of saline solution.

It appears from this experiment (table IV), that nearly all the peptone fractions interfere with the hemolytic properties of the amboceptor, but some more than others. Though the interference with the hemolysis may be in part due to the simultaneous absorption of complement, the inhibition is principally exerted on the amboceptor. As already stated, all the peptone fractions interfere with the hemolysis; the peptone mixture (Witte) does not. The hemolysis that takes place is more marked in the higher dilutions.

TABLE IV.

Mixtures of Peptone Fractions with Immune Amboceptor.

Hemolysin Dilutions.	1:250	1:500	1:750	1:1,000
Corresponding peptone dilutions	5 per cent.	2.5 per cent.	1.66 per cent.	1.25 per cent.
Peptone (Witte)	++[6]	+++	o	o
Protalbumose (Witte)	o	+	++	+++
Heteroalbumose (Witte)	+	+	++	+
Deuteroalbumose A (Witte)	+	++	+	o
Deuteroalbumose B (Witte)	o	++	+	o
Peptone A (Witte)	+	+	+	o
Peptone B (Witte)	+	++	++	o
Protalbumose (casein)	o	++(?)	++(?)	+(?)
Deuteroalbumose A (casein)	+	+	+	o
Deuteroalbumose B (casein)	+	++	+	o
Peptone A (casein)	o	+	++	o
Peptone B (casein)	+	+++	++	+

Detail.—0.5 c.c. of hemolysin (1:5 dilution) and 0.5 c.c. of peptone fractions (10 per cent.) were incubated for one hour at 37° C. in the thermostat, then diluted so as to give dilutions of hemolysin 1:250, 1:500, 1:750, and 1:1,000, respectively.

Tests were made by using in each tube 0.2 c.c. of each dilution of hemolysin, 0.2 c.c. of 5 per cent. red blood cells, 0.2 c.c. of complement (guinea pig), and 0.4 c.c. of saline solution.

This may be accounted for in one of two ways; either that on diluting the peptone-amboceptor mixtures, the inhibitory effect of the peptone disappears before the hemolytic action; or, that not all of the hemolytic amboceptor is neutralized by the peptone. The latter possibility seems more likely, because the hemolytic efficiency of the amboceptor treated with the peptones is, in higher dilutions, considerably less than that observed with the corresponding dilutions of the amboceptor alone in the control tests.

In view of the effects produced upon the red blood cells and immune serum elements by the different peptone fractions, it seemed necessary to inquire whether red blood cells exposed to the action of or sensitized with the peptone fractions, would show marked changes in their behavior towards immune amboceptor and complement.

Experiments were carried on along the following lines:

1. Red blood cells, sensitized with peptone fractions, were kept for one hour at 37° C., and were then washed free of the peptone, and to the suspensions of the washed cells were added (*a*)

[6] The degree of hemolysis is indicated by +.

10 per cent. complement alone, (*b*) 1:1,000 amboceptor alone, and (*c*) 10 per cent. complement + 1:1,000 amboceptor.

2. Red blood cells were sensitized with immune amboceptor (1:1,000 dilution) for one hour, then the cells were washed free of the amboceptor, and to their suspension were added (*a*) 10 per cent. complement alone, and (*b*) different peptone fractions.

3. Red blood cells were sensitized with mixtures of equal parts of peptone and amboceptor (1:500 dilution) at the same time, the cells were incubated for one hour at 37° C. and were washed free of the peptone hemolytic mixtures, and to the suspension of the sensitized cells, 10 per cent. complement was added (table III).

The results of these three experiments show that red blood cells impregnated with peptone fractions are not affected by amboceptor or complement alone, but in the presence of the two, hemolysis occurs in a normal manner. The cells impregnated with deuteroalbumose A (Witte) 10 per cent., deuteroalbumose A (casein) 10 per cent., heteroalbumose (Witte) 1 per cent., and deuteroalbumose B (Witte) 1 per cent., seem to be altered so that the hemolysis is hindered to a certain extent.[7]

SUMMARY.

The experiments here described indicate that the peptone fractions can alter the different blood elements in such a manner as to affect their immunological reactions. This power is not possessed by all the peptones equally, either qualitatively or quantitatively. If we regard the phenomenon of hemolysis by immune serum in the light of a biochemical process, depending upon properties inherent in certain constituents of the serum and the red blood cells, the experiments described reveal a number of facts concerning the immunochemical action of albumoses and peptones. These substances, which depend for their separation and identification upon purely physical means, show differences in their behavior toward the elements concerned in the production of hemolysis.

[7] One observation, made repeatedly upon cells impregnated with the peptone fractions and kept in the ice-box over night, showed that these, when exposed to amboceptor and complement action, dissolved more readily than those in control tests. This may bear some analogy to the observations of Landsteiner on the hemolysis of blood from cases of paroxysmal hemoglobinuria.

Inagaki (7) has shown that the albumoses can combine with nucleohiston. It is not improbable, therefore, that some of the reactions are chemical as well as physicochemical in character.

Further studies are necessary to determine whether the reactions elicited can aid us in the differentiation of the peptone bodies, for which at present we possess but few tests. The results, however, are suggestive, and may aid in recognizing the presence of these substances in blood serum.

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EXPERIMENTAL STUDIES ON THE ADMINISTRATION OF SALVARSAN BY MOUTH TO ANIMALS AND MAN.*

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PLATE 70.

The experimental studies here described were undertaken with the view of determining whether salvarsan could be administered by mouth without producing poisonous effects, and whether, when so given, it exerted a therapeutic influence on the manifestations of syphilis. Certain studies incidental to the oral administration of salvarsan have also been made which may prove not to be devoid of interest.

ANIMAL EXPERIMENTATION.

Before any attempt was made to administer salvarsan to patients by mouth, the drug was given to experimental animals to determine whether or not it would produce deleterious effects. It was also necessary to prove that the medicament thus given was absorbed.

In administering the drug by mouth to animals, we sought to determine four points: (1) the primary and secondary toxic effects, if any, of a single large dose and of several small doses of the medicament; (2) the presence or absence of arsenic in the bile and urine and in the stomach and intestinal contents; (3) the influence of salvarsan upon the bacterial flora of the intestinal tract, especially upon that of the colon; (4) the effect of the drug upon the parenchymatous organs, especially those concerned in elimination.

TOXIC EFFECTS.

To determine the primary effects of salvarsan given by mouth, the drug was administered in various forms to a series of cats,

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rabbits, and dogs. It was given in pills, in capsules, and dissolved in milk and water.

Experiment 1.—A cat, weighing 2,650 gm., was given 0.01 gm. of salvarsan dissolved in 10 c.c. of milk and water. No other food was given for twenty-four hours. There was neither vomiting nor diarrhea The cat was killed at the end of twenty-four hours for further study.

Experiment 2.—A cat, weighing 3,250 gm., was given 0.01 gm. of salvarsan dissolved in 10 c.c. of warm milk and water. No other food was given. The animal showed no toxic effects and was killed and autopsied forty-eight hours later.

Experiment 3.—A cat, weighing 2,550 gm., received 0.05 gm. of salvarsan dissolved in 10 c.c. of milk and water. The animal showed no toxic effects.

Experiment 4.—A cat, weighing 850 gm., received 0.05 gm. of salvarsan in a capsule. The animal was autopsied twenty-four hours later, but showed no evidence of toxic action.

Experiment 5.—A cat, weighing 2,700 gm., received 0.05 gm. of salvarsan in a capsule. The animal showed no evidence of toxic effects and was autopsied twenty-four hours later. The findings were negative.

Experiment 6.—A rabbit, weighing 2,080 gm., received 0.05 gm. of salvarsan dissolved in 20 c.c. of milk and water. The animal showed no evidence of toxic effects and was killed eighteen hours later and autopsied.

Experiment 7.—A rabbit, weighing 2,000 gm., received 0.05 gm. of salvarsan dissolved in 12 c.c. of milk and water. The animal showed no evidence of toxic effects and was autopsied at the end of forty-eight hours.

Experiment 8.—A rabbit, weighing 1,380 gm., received 0.05 gm. of salvarsan dissolved in 12 c.c. of milk and water. The animal showed no toxic effects, and was autopsied seventy-two hours later.

Experiment 9.—A rabbit, weighing 1,450 gm., received 0.05 gm. of salvarsan in a capsule. The animal showed no toxic effects, and was autopsied twenty-four hours later with negative findings.

Experiment 10.—A rabbit, weighing 1,400 gm., received 0.05 gm. of salvarsan in a capsule. The animal showed no toxic effects and was autopsied forty-eight hours later. The findings were negative.

Experiment 11.—A rabbit, weighing 1,350 gm., received 0.008 gm. of salvarsan in a capsule. The animal was autopsied ninety-six hours later. It had shown no toxic effects, and the autopsy findings were also negative.

Experiment 12.—A rabbit, weighing 1,400 gm., received 0.008 gm. of salvarsan in a capsule. The animal was autopsied ninety-six hours later. It had shown no toxic effects, and the autopsy findings were also negative.

Experiment 13.—A cat, weighing 2,550 gm., received 0.05 gm. of salvarsan dissolved in 10 c.c. of milk. The animal showed no primary toxic effects and at the present time is apparently in a normal condition.

Experiment 14.—A cat, weighing 3,550 gm., received 0.02 gm. of salvarsan dissolved in 20 c.c. of warm milk. The animal did not show any primary toxic effects and at the present time apparently is in good health.

Experiment 15.—A cat, weighing 2,850 gm., received 0.04 gm. of salvarsan dissolved in 40 c.c. of warm milk. The animal showed no toxic effects and was autopsied forty-eight hours later.

Experiment 16.—A dog, weighing 5,700 gm., received 0.025 gm. of salvarsan in the form of a pill. No toxic effects were seen, and the dose was repeated in twenty-four hours without producing any deleterious effects.

Experiment 17.—A dog, weighing 7,000 gm., received a pill containing 0.025 gm. of salvarsan and this was followed by 10 c.c. of milk. The dose was repeated at the end of twenty-four and forty-eight hours without producing any appreciable toxic effects.

Experiment 18.—A dog, weighing 7,200 gm., received in the form of a pill 0.01 gm. of salvarsan every day for twenty-two days, followed by 0.05 gm. in a capsule each day for the next seven days. The animal did not lose weight, its appetite and spirits remained perfectly normal, and at no time were toxic effects seen.

These experiments show that salvarsan is without immediate toxic effects when administered to animals by mouth in a relatively large single dose or in multiple smaller doses. In some of these experiments six weeks have elapsed since the drug was given, and the animals have shown no late or secondary poisonous effects attributable to arsenic. The solutions were made and administered while fresh, without the addition of an alkali.

Many of the pills had been made up as long as fifteen days before administration and had become very dark in color and were quite hard. These were crushed in a mortar before administration but in no instance could harmful effects be noticed.

The capsules, which contained diatomaceous earth with the salvarsan, did not undergo any change of color although exposed to light for several weeks.

ABSORPTION AND ELIMINATION.

The intravenous and intramuscular administration of salvarsan are followed by the elimination of arsenic in the urine and feces. So far there are no reliable tests for salvarsan *per se* in any of the excretions. The question of absorption of salvarsan from the intestinal tract is worthy of study. Whether or not the drug undergoes marked changes in the intestinal canal is as yet undetermined. We have examined the secretions of a number of experimental animals in order to determine whether the drug was being eliminated as arsenic. If arsenic were found, this would indicate absorption of the drug under these conditions.[1]

[1] We are indebted to Professor Alonzo Taylor and Dr. Rinker, of the Department of Physiological Chemistry of the University of Pennsylvania, for assistance and suggestions in conducting these tests.

Technique.—All tests were qualitative. Suitable controls were used. In brief, the material (stomach contents, feces, bile, and urine) were oxidized with concentrated nitric and sulphuric acids, after which sodium and potassium nitrate were added and the whole mass was fused with more of the same salts. The resulting product was dissolved in concentrated sulphuric acid over a water bath until all nitric acid had disappeared. The March test was then employed.

The results are given in the following table.

TABLE I.

Tests for Arsenic.

No. of experiment.	Animal.	Dose in gm.	Time of removal after administration.	Product.	Arsenic present.
3	Cat	0.05	24 hours	Stomach	Well defined trace
				Feces	Well defined trace
				Bile	Trace
				Urine	Trace
6	Rabbit	0.05	24 hours	Stomach	Well defined trace
				Feces	Well defined trace
				Bile	Trace
				Urine	Trace
7	Rabbit	0.05	48 hours	Stomach	No trace
				Feces	Trace
				Urine	Very slight trace
8	Rabbit	0.05	72 hours	Stomach	No trace
				Feces	No trace
				Bile	No trace
				Urine	No trace
18	Dog	0.01	After 3 doses	Feces	Well defined trace

These results show that within twenty-four hours after the oral administration of salvarsan, arsenic may be found in the bile and urine. Its presence in the stomach contents and feces may be indicative of failure of absorption. At the end of forty-eight hours, the quantities of arsenic found were very minute, and after seventy-two hours none was found in the stomach, feces, bile, or urine. It is probable that only a portion of the salvarsan was absorbed from the intestinal canal and that this was quickly eliminated largely in the form of arsenic.

INFLUENCE ON INTESTINAL BACTERIA.

As a matter of additional interest, a quantitative examination of the bacteria of the gastro-intestinal canal was made in a few of the

experimental animals, since arsenic and possibly some of the unchanged drug had been found in the feces twenty-four hours after the oral administration of salvarsan.

Technique.—For quantitative work with intestinal bacteria, the question of a satisfactory technique was most important. The animals were usually kept from all food for twenty-four hours before and after the administration of salvarsan. As a result, the stomach was usually empty at autopsy. Cultures were made of the stomach, ileum (close to the ileocecal valve), and the upper portion of the rectum. After asepticizing the gut with a hot iron, the wall was cut through and cultures were secured by rubbing the wall or contents with a sterilized platinum loop which measured four millimeters in diameter. The loop was then thoroughly shaken in 10 cubic centimeters of sterilized salt solution. This solution was thoroughly emulsified and 0.5 of a cubic centimeter, one cubic centimeter, and two cubic centimeters were plated with 10 cubic centimeters of plain neutral agar. The plates were incubated at 37° C. for twenty-four hours and then kept at room temperature until the end of the forty-eighth hour, when counts were made. The control animals were living under exactly the same conditions and receiving the same diet. From them likewise food was kept for forty-eight hours preceding the autopsy and the taking of the cultures.

While the number of cats from which cultures were made was comparatively small, the results were fairly constant. The most

TABLE II.

The Results of Quantitative Bacteriological Examination of the Intestinal Tract of Animals Receiving Salvarsan by Mouth.

No. of experiment.	Animal.	Dose in gm.	No. of hours separating the administration of the drug and the taking of cultures.	Organ.	No. of colonies of bacteria per c.c. of emulsion.
1	Cat	0.01	24	Stomach	10
				Ileum	450
				Colon	14,000
3	Cat	0.05	24	Stomach	None
				Ileum	270
				Colon	10,500
15	Cat	0.04	48	Stomach	18
				Ileum	1,200
				Colon	17,000

TABLE III.

Table of Control Bacteriological Examinations.

No. of control.	Animal.	Organ.	No. of colonies of bacteria per c.c. of emulsion.
1	Cat	Stomach	24
		Ileum	11,000
		Colon	19,000
2	Cat	Stomach	19
		Ileum	12,200
		Colon	20,000
3	Cat	Stomach	50
		Ileum	17,000
		Colon	19,500

marked influence was seen in the lower end of the ileum, where, within twenty-four hours after the administration of salvarsan, the total number of bacteria was found much decreased. In the colon, the decrease in the number of bacteria was about 30 per cent. At the end of forty-eight hours, however, the differences were less marked.

EFFECTS OF SALVARSAN ON PARENCHYMATOUS ORGANS, ESPECIALLY THOSE OF ELIMINATION.

Urinary Examinations.—The urine of the six animals that received salvarsan by mouth, Nos. 4, 5, 9, 10, 11, and 12, and of four others, Nos. 19, 20, 21, and 22, that received the drug intravenously, was examined, but in no instance was the presence of albumin or casts demonstrated, the findings being, in fact, entirely similar to those in the normal controls. In all the animals mentioned, the special test for salvarsan in the urine advocated by Abelin was tried. In these tests, we secured a clear dark brownish discoloration in all specimens of urine from the experimental and control animals, but in no instance did we find the red color described as a positive reaction in this test.

Autopsy Protocols.—Twelve of the eighteen experimental animals were autopsied at intervals varying from eighteen to ninety-six hours after the administration of salvarsan. In none of these animals were there any gross evidences of deleterious effects. The heart, lungs, liver, spleen, kidneys, and the mucosa of the stomach and of the entire intestinal tract were found to be normal. The liver and kidneys of many of the animals were examined in the

fresh condition for albuminous degeneration of the cells, but the findings did not differ from those of the control animals. The liver of one cat (experiment 4) had the gross appearance of fatty changes, but a control animal showed an exactly similar condition.

Histological Examination.—Sections of the heart, lungs, liver, spleen, kidneys, stomach, and ileum from experimental animals 1, 2, 4, 5, 9, 10, 11, and 12 were fixed, stained, and examined histologically for evidence of vascular disturbances and degenerative changes. After examining the sections from animals 1, 2, 4, and 5, which were cats, we at first thought that, in addition to the fatty infiltration found normally in a large proportion of cats, important albuminous and fatty degenerative changes had been produced. But after studying the organs of three control cats living under the same conditions, it was found that no degenerative or vascular changes could be ascribed to the influence of the salvarsan.

In order to study this question more thoroughly, salvarsan was administered intravenously in alkaline solution to the following four rabbits:

Experiment 19.—A rabbit, weighing 1,500 gm., received intravenously 0.006 gm. of salvarsan dissolved in 16.8 c.c. of water. The animal was autopsied forty-eight hours later.

Experiment 20.—A rabbit, weighing 2,500 gm., received intravenously 0.01 gm. of salvarsan dissolved in 28 c.c. of water. The animal was autopsied ninety-six hours later.

Experiment 21.—A rabbit, weighing 1,400 gm., received intravenously 0.0056 gm. of salvarsan dissolved in 15.6 c.c. of water. It was autopsied eighteen hours later.

Experiment 22.—A rabbit, weighing 1,000 gm., received 0.004 gm. of salvarsan dissolved in 11.2 c.c. of water, and was autopsied twenty-four hours later.

According to body weight, the doses administered were about equal to those administered intravenously to man. None of these animals showed any toxic effects whatsoever, the urine examinations were negative, and the gross autopsy findings were entirely negative. Sections of the heart, lungs, liver, kidneys, spleen, stomach, and ileum of these four rabbits and of rabbits 9, 10, 11, and 12, were examined histologically and contrasted with preparations of the organs from our normal rabbits. While the sections from the hearts showed in some instances a loss of muscle striations and fragmentation of the muscle cells with slight fatty

changes, entirely analogous changes were found in the controls. The lungs showed no changes. The stomach and ileum appeared entirely normal, there being no evidences of vascular changes or degeneration of cells. Sections of the liver showed in many instances marked granularity of the cells with perivascular interstitial connective tissue proliferation, but in the normal controls entirely analogous conditions were found. The kidneys showed some cloudy swelling of cells in a few of the sections, but no vascular changes. The controls were similar. The spleen showed in a few instances a large number of eosinophil cells and occasionally apparent enlargement of the Malpighian bodies. There was no congestion or endothelial cell proliferation. The normal controls were similar in all particulars.

As a result of these studies, we must conclude that salvarsan in the doses in which we administered it does not produce appreciable degenerative changes in the principal organs within ninety-six hours following its administration.

ORAL ADMINISTRATION OF SALVARSAN TO MAN.

As it had been demonstrated that salvarsan could be given by mouth to cats, dogs, and rabbits in relatively large doses without producing toxic effects, or, indeed, untoward symptoms of any kind, we proceeded to administer it to human subjects.

A decigram of salvarsan dissolved in hot milk was first given to three men suffering from secondary and tertiary syphilis. In two of these patients the drug was well borne, no symptoms of gastro-intestinal irritation and no other disturbing symptoms developing.

Case 1.—In a man thirty years old, vomiting, followed by diarrhea, occurred two hours after the ingestion of the salvarsan, but the patient had no pain and was not otherwise disturbed.

On the following day, a second dose of the same amount was administered to this patient without producing vomiting or diarrhea. Four days later, 0.2 gm. of salvarsan dissolved in hot water to which a little peppermint water was added, was given. This was followed by some vomiting and diarrhea, but the patient had no pain and did not feel ill in any way. On the two succeeding days, he received 0.2 gm. and 0.15 gm., respectively, without vomiting or diarrhea.

This patient had an extensive small papular syphilide (follicular syphilide), for which he had a short time before received nine mercurial inunctions, with but slight influence on the eruption. A mucous patch on the lip healed promptly after the salvarsan was given and the eruption was perceptibly improved.

Case 2.—A man, twenty years old. He had a chancre and a macular syphilide with beginning papular lesions.

On January 21, 1912, the patient was given 0.1 gm. of salvarsan in 90 c.c. of milk. On January 20, the dose was repeated. On January 24, 0.2 gm. was given in water with peppermint. On January 25, 0.2 gram was again administered.

All of these doses were well borne, the patient experiencing no unpleasant symptoms whatsoever. The treatment was discontinued owing to a misunderstanding of orders.

During the administration of the drug, the macular eruption disappeared, but a large flat papular syphilide continued to make its appearance.

Case 3.—The patient was a man, thirty-eight years old. He has been a hard drinker. Twelve years ago he had a chancre and secondaries, and then had received mercurial inunctions, but in the past twelve years he has received no treatment. The patient has a large ulcerating gumma below the knee which is deeply excavated and discharges profusely (figure 1). The gumma has existed since September, 1911.

Treatment.—On January 19, 1912, and again on January 20, 0.1 gm. of salvarsan was administered in milk. Healthy granulations were observed on January 20. On January 25, 0.2 gm. of salvarsan was administered in water, and on this date the first photograph (figure 1) was taken. On January 26, 0.3 gm. of salvarsan was given in water. On January 27, the gumma was healing quickly, and granulations were appearing rapidly. On the 27th, the 28th, and again on the 29th of January, a capsule containing 0.1 gm. of salvarsan and 0.2 gm. of diatomaceous earth was given.

On February 2, the ulcer was about half its former size. The granulations were on a level with the skin. The discharge was very scanty, and cicatrization from the border was advancing. The local treatment consisted merely of the employment of a wet boric acid dressing. The patient stated that he felt well and that his appetite was most keen.

On February 2, the full dose of 0.6 gm. of salvarsan in 120 c.c. of water flavored with peppermint was administered. No unpleasant symptoms followed. The ulcer was healing rapidly.

On February 10, another full dose of 0.6 gm. of salvarsan was given. No unpleasant symptoms followed.

The second photograph (figure 2), was taken on March 1, just five weeks after the first. It shows the ulceration to be practically healed although epidermization is not yet complete.

Case 4.—A young woman, with a recurrent papulosquamous syphilide of the wrists and palms, received 0.1 gm. of salvarsan in a capsule, once a day for ten days. The drug was well borne, but as the improvement was but slight, intravenous administration was resorted to.

Case 5.—A young negro, with a beginning follicular syphilide on the trunk and an annulo-papular syphilide of the penis and scrotum, received the full dose of 0.6 gm. of salvarsan in 200 c.c. of water. About three quarters of an hour later he vomited, and after this had seven loose bowel movements.

It was subsequently ascertained that prior to the ingestion of the drug, he had had no food for eighteen hours. Three days later, the follicular syphilide

had almost disappeared, but the patches on the penis and scrotum were but slightly influenced.

A week after the first administration, a second dose of 0.6 gm. of salvarsan in 200 c.c. of water was given. This was followed by slight nausea, but no vomiting. The patient had four loose bowel movements, but no soreness or pain.

After the second dose of salvarsan, the genital eruption improved but did not disappear. Other treatment was therefore substituted.

Case 6.—The patient was a woman, forty years old, who had on her back a palm-sized tubercular syphilide that had come on three years after infection. She received four capsules of 0.1 gm. of salvarsan on successive days, and three days later took 0.6 gm. dissolved in 200 c.c. of water. The patient had two loose bowel movements, but no vomiting. There was practically no improvement in the eruption.

Case 7.—A mulatto girl, aged nineteen years, with an extensive pustular syphilide of the varicelliform type, was given 0.6 gm. of salvarsan in 200 c.c. of water. Three hours later she vomited twice, and had some cramps, but no diarrhea. Three days later, 0.6 gm. of salvarsan was again administered. Some hours later the patient had nausea and slight vomiting.

The influence of the salvarsan on the eruption in this patient was extremely slight.

Urinary Examinations.—Twenty-four hours after the full doses of salvarsan had been administered by mouth, the urine of the patients treated by mouth was subjected to careful chemical and microscopical examination. Albumen was tested by the nitric acid, heat, and potassium ferrocyanid tests and was absent in all the examinations except one in which a trace was disclosed by the potassium ferrocyanid reaction only. In this specimen of urine, a single hyaline cast was found. With this exception, the microscopical examinations were negative.

SUMMARY.

Salvarsan in capsule and in solution in doses varying from 0.1 to 0.6 of a gram was administered by mouth to seven patients suffering from syphilis. Five patients were given full doses of 0.6 of a gram. One patient (case 3) received in the aggregate within four weeks 2.4 grams without loss of weight or any disturbing effects. An ulcerating gumma of the knee was practically healed after a period of five weeks. This was the only patient of the seven, however, in whom notable improvement in the cutaneous manifestations followed the administration of the drug. This ulceration

would probably have healed as soon, or possibly sooner, under the influence of mercury and the iodids.

From the above observations, it would appear that salvarsan administered by mouth up to the full dose of 0.6 of a gram, has a perceptible though feeble therapeutic effect on cutaneous syphilitic manifestations. We should naturally expect the influence of any drug to be more evident in the case of a gumma, in which spirochætes are only sparsely present, than in the secondary syphilides in which the spirochætes are present in enormous numbers. Cutaneous gummata are readily healed at times by very small doses of mercury or iodids.

It is interesting to note that the administration of the full dose of the drug by mouth does not appear to give rise to symptoms of systemic arsenical poisoning, unless the vomiting and diarrhea be regarded as such. After some of the full dose administrations, there was neither vomiting nor diarrhea. As salvarsan contains 34 per cent. of arsenic, these patients received the equivalent of five grains of arsenic. In case 3, in the course of four weeks the patient took by mouth the equivalent of over twelve grains of arsenic, without vomiting or having diarrhea, and with no loss of weight or disturbance of health. The urine of this patient was entirely normal. It would seem, therefore, that salvarsan is not converted in the gastro-intestinal tract into an organotropic arsenical compound, and it is possible that it may remain practically unchanged.

CONCLUSIONS.

1. Salvarsan can be administered in pills, in capsules, and in solution to lower animals in dosage of 0.02 to 0.03 of a gram per kilo of body weight, without producing toxic symptoms. A dog received 0.57 of a gram of salvarsan in pill and capsule form in the course of twenty-nine days without any disturbing effects.

2. After the oral administration of salvarsan, arsenic is found in the bile and urine at the end of twenty-four hours, but it disappears by the end of seventy-two hours.

3. Twenty-four hours after the oral administration of salvarsan to cats, the number of bacteria in the intestinal tract appears to be

FIG. 2.

FIG. 1.

reduced, the reduction being most striking in the lower end of the ileum.

4. Salvarsan administered by mouth and likewise intravenously to rabbits in doses approximating those employed in human subjects, does not produce, at least within ninety-six hours following its administration, any appreciable microscopic changes in the important viscera.

5. With the exception of a little vomiting and diarrhea in some cases, salvarsan can be given by mouth up to 0.6 of a gram to human subjects without producing toxic symptoms. Thus administered, the drug exerts a therapeutic influence, but this influence is too feeble to warrant its use by this route.

EXPLANATION OF PLATE 70.

FIG. 1. Case 3. First photograph, taken January 25, 1912. Gummatous ulcer developing twelve years after infection. No treatment was given after the initial inunctions. This patient received 2.4 grams of salvarsan by mouth within a period of four weeks.

FIG. 2. Case 3. Second photograph, taken March 1, 1912.

THE PRODUCTION OF FOREIGN BODY GIANT CELLS IN VITRO.*

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PLATES 71–74.

In a recent paper,[1] the effect of the addition of foreign bodies to cultures of certain tissues of the chick embryo was briefly discussed. It is the object of the present communication to describe in some detail these phenomena, which relate chiefly to the formation of foreign body giant cells, with a discussion of their possible significance.

Technique.—For the preparation of the tissue cultures, Burrows'[2] original method has been employed. This consists in suspending teased fragments of tissue, 0.5 to one millimeter in diameter, in hanging drops of plasma, cover-glasses and relatively deep hollow ground slides being used for this purpose. The method of adding the foreign bodies, upon which a successful outcome largely depends, will be given in detail.

Lycopodium spores were selected for use as the foreign objects, their diameter, forty microns, rendering them especially suitable. That is, they were found to be too large to be engulfed by phagocytic cells, and yet sufficiently small to be easily surrounded by wandering cells. Furthermore, their characteristic architecture and refractivity made them easily recognizable even when embedded in a mass of cells. In the form of the commercial "yellow powder," they do not form readily a homogeneous suspension in water or watery solutions. This difficulty may, however, be easily overcome by first moistening the powder with alcohol and then adding water. The alcohol is subsequently removed by boiling. Small drops of

* Received for publication, March 14, 1912.

[1] R. A. Lambert, *Anat. Rec.*, 1912, vi, 91.

[2] M. T. Burrows, *Jour. Exper. Zoöl.*, 1911, x, 63.

this aqueous suspension are placed on cover-glasses and allowed to dry. The thickness of the suspension and the size of the drops should be such that the spores are left widely scattered over the surface. These cover-glasses are used for the hanging drop preparations, care being taken in adding the tissue and plasma to leave, as far as possible, the spores adherent to the cover-glass.

In a majority of the experiments, spleen tissue from twenty day chick embryos and from one day chicks was used. Before discussing the phenomena associated with the addition of foreign bodies, it may be well to describe briefly the character of the outgrowth in unmodified spleen cultures.

After a few hours' incubation, small mononuclear and polynuclear leucocytes begin to wander from the piece of tissue. Larger cells with very irregular cell bodies and numerous processes follow shortly. During the two succeeding days these cells may spread to the very edge of the drop of plasma. At this time the leucocytes become rounded or very ragged in form, and in stained preparations show pyknosis and fragmentation of nuclei (figure 3). The larger cells, though early showing numerous fat droplets in their cytoplasm, may remain active from ten to twelve days.

A group of cells showing these two types is seen in figure 3, which is a drawing from a small field near the edge of the drop of plasma in a two day culture. The leucocytes show pyknotic and fragmented nuclei. The larger cells vary considerably in form, some being spindle or triangular in shape, others tending to preserve a round or oval outline. There is no striking difference in the character of the nuclei.

That this group of larger cells includes both endothelial cells and large pulp cells, seems probable, inasmuch as each possesses the power of ameboid wandering. On account of the large accumulation of fat droplets in all of these cells, the use of protoplasmic stains which might serve for finer differentiation, is not practicable.

A third type of cell is usually observed in spleen cultures on the second or third day of incubation. The cells of this type appear in the form of long sprouting outgrowths from the central masses of tissue. In their further growth, they form radiating strands of cells. In stained preparations, these are seen to be typical young connective

tissue cells. After five to six days' incubation, large multinuclear giant cells are occasionally seen spread out in a thin sheet on the cover-glass. The process of formation and significance of these giant cells will be discussed in a later paragraph.

Cultures to which lycopodium spores have been added present usually by the second day of incubation a rather striking picture, if conditions have been favorable for an active outwandering of cells. Many of the spores are seen surrounded by dense masses of wandering cells (figures 1 and 2). Further observation of such a mass shows a rather rapid fusion of the cells immediately surrounding the spore, resulting in the formation of a thick giant cell, in which the enclosed spore may be quite invisible. They contain, as a rule, numerous fat droplets and often large vacuoles also. A distinct cell membrane is seen. Protoplasmic processes may be thrown out, particularly if the spore is adherent to the cover-glass.

Preparations of these giant cells stained *en masse* show very well their general character, and if the cells are not too thick, the character of the nuclei can also be determined. Paraffin sections, however, are necessary for more detailed studies. A section through such a giant cell is shown in figure 4. The nuclei in some of these giant cells are of two types: one small, deeply staining, and rather pyknotic; the other larger and rather vesicular. They correspond to the nuclei in the two types of wandering cells described above; that is, to the mononuclear leucocytes, and to the larger irregularly shaped cells which we are inclined to interpret as a group including both endothelial cells and large spleen pulp cells.

Giant cells are also formed about the spores attached to the central mass of tissue. As a result of the flattening and consequent diminution in thickness of this piece of tissue, gross changes taking place in it can often be readily observed in the living cultures.

Observations on the reaction about the spores is made still easier by previously staining them. The tissue may, in addition, be stained with some vital stain. An excellent contrast is obtained, for example, by treating the spores with methylene blue, and the tissue with neutral red, the spores being previously stained and washed and the vital stain added to the fluid plasma before using.

It is possible, however, without such color contrasts to observe in the compact but translucent piece of tissue the formation of dark

rings about the spores. This phenomenon is often observed about the fifth or sixth day, but it is usually more marked in cultures seven to nine days old.

To insure a period of activity of the tissue longer than six days, we have found it advisable to make transfers to fresh plasma about the fifth day. This can be readily accomplished, as we have shown in an earlier report.[3]

Figure 5 shows a section through a piece of spleen which presented in the fresh state a spore dimly visible surrounded by a dark ring. In the stained section, the ring is seen to be a giant cell which does not differ in its general character from a foreign body giant cell formed in the animal body.

From the description of spleen cultures given above, it is obvious that in the formation of the giant cells in the zone of wandering cells, only wandering cells can be concerned. In the case of those giant cells formed in the mass of tissue, however, it is not so easy to draw conclusions. The question resolves itself into a determination of the rôle played by leucocytes, connective tissue cells, pulp cells, and endothelial cells.

The fact that leucocytes remain active in cultures for only two or three days, and that these giant cells are found, as a rule, after this time, removes, we think, this group of cells from serious consideration. The character of the nuclei in the giant cell throws some light on the question. They are rather large, somewhat vesicular and resemble closely the nuclei in the adjacent spleen cells. The architecture of the organ, however, at this stage is so disturbed that fine distinctions in regard to the character of these cells cannot well be made. For this reason further experiments were resorted to which it appeared might settle definitely the question as to whether connective tissue cells played a part in the process.

These experiments consisted in the addition of lycopodium spores to cultures of the heart of chick embryos, where the outgrowth consists of diffuse connective tissue. In no instance was there observed any tendency on the part of the connective tissue either to surround the foreign bodies or to form a giant cell.[4]

[3] R. A. Lambert and F. M. Hanes, *Jour. Exper. Med.*, 1911, xiii, 505.

[4] Further experiments are being undertaken for the purpose of determining more definitely what cells take part in the formation of these foreign body giant

It should be mentioned in this connection that we have observed in spleen cultures the same phenomena associated with foreign bodies other than lycopodium spores. For example, figure 6 shows a piece of cotton fiber enclosed in a large giant cell. A distinct cell membrane and protoplasmic processes can be seen. The stages in the formation of this giant cell from a mass of wandering cells were observed.

In an earlier paragraph, reference was made to the occurrence of large flat giant cells in cultures of spleen to which foreign bodies had not been added. Giant cells of this type make their appearance from the fourth to the eighth day of incubation. They contain from ten to several hundred nuclei, and measure 200 microns to a millimeter in diameter. As a rule, they are so thinly spread out on the cover-glass that they do not exceed in thickness ordinary mononuclear cells (figure 7). Fat droplets of remarkably large size accumulate in their cytoplasm. They are found usually about the original piece of tissue, either around the border or in the zone of attachment to the cover-glass. It also happens, as we indicated above, that the giant cells which are formed from aggregations of cells about lycopodium sometimes spread themselves out in a thin sheet, if the foreign body is adherent to the cover-glass. These observations on the constant relation of giant cells of this type to the cover-glass, or to the cover-glass and some introduced foreign body have led us to suggest that we are probably dealing here also with a true foreign body giant cell, the cover-glass acting as the foreign body. The fact that such giant cells are found so frequently in the zone of attachment of the original piece of tissue to the cover-glass, that is, in an area where numerous wandering cells are massed together on one side of a foreign object, would favor this conception.

CONCLUSIONS.

1. Foreign body giant cells may be produced *in vitro* by the addition of foreign objects such as lycopodium spores and cotton fibers to cultures of chick embryo spleen.

cells. Several investigators have shown that in certain parts of the body a single type of cell may be concerned in the formation of such structures. Marchand (*Deutsch. Chir.*, 1901, xvi, 352), for example, found that the giant cells that enclosed lycopodium spores placed in the peritoneal cavity of rabbits were formed almost entirely out of the endothelial cells covering the serosa.

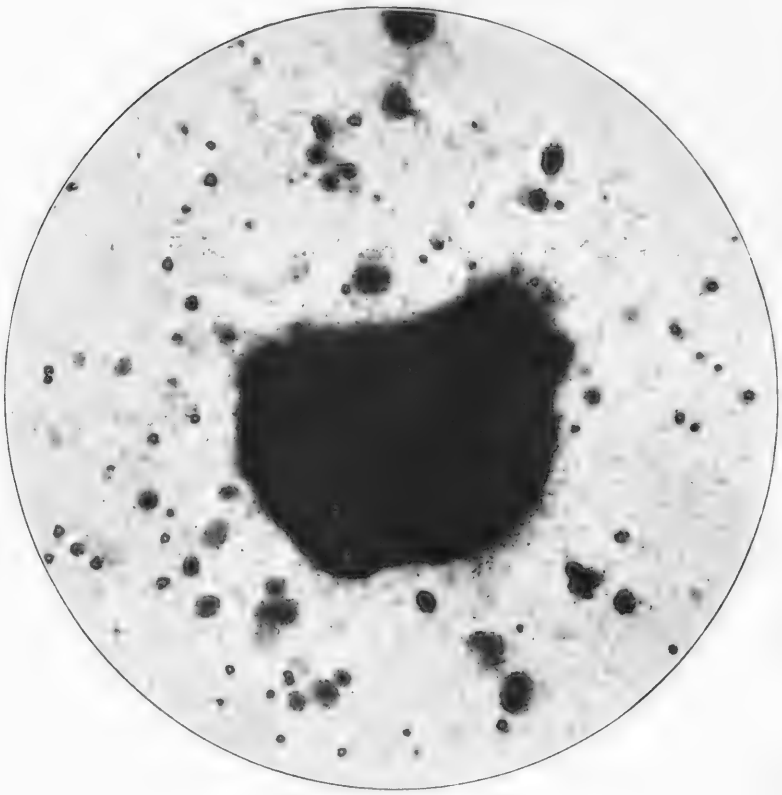

FIG. 1.

FIG. 2.

FIG. 3.

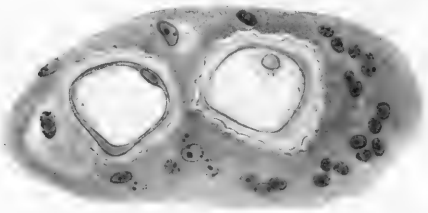

FIG. 4.

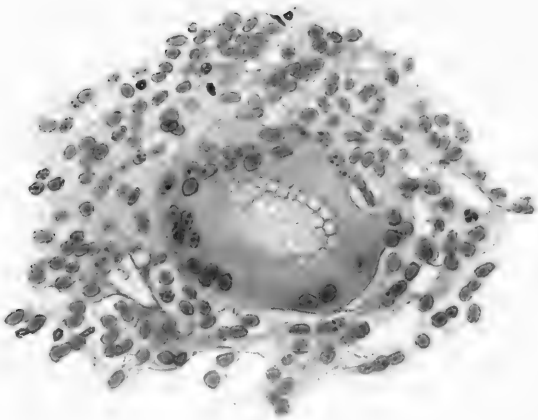

FIG. 5.

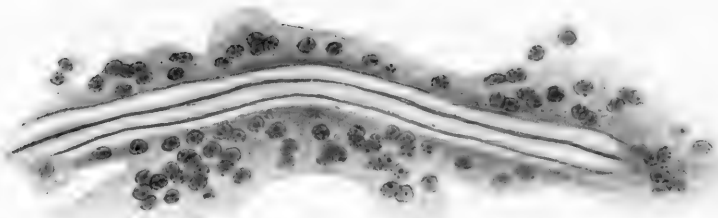

FIG. 6.

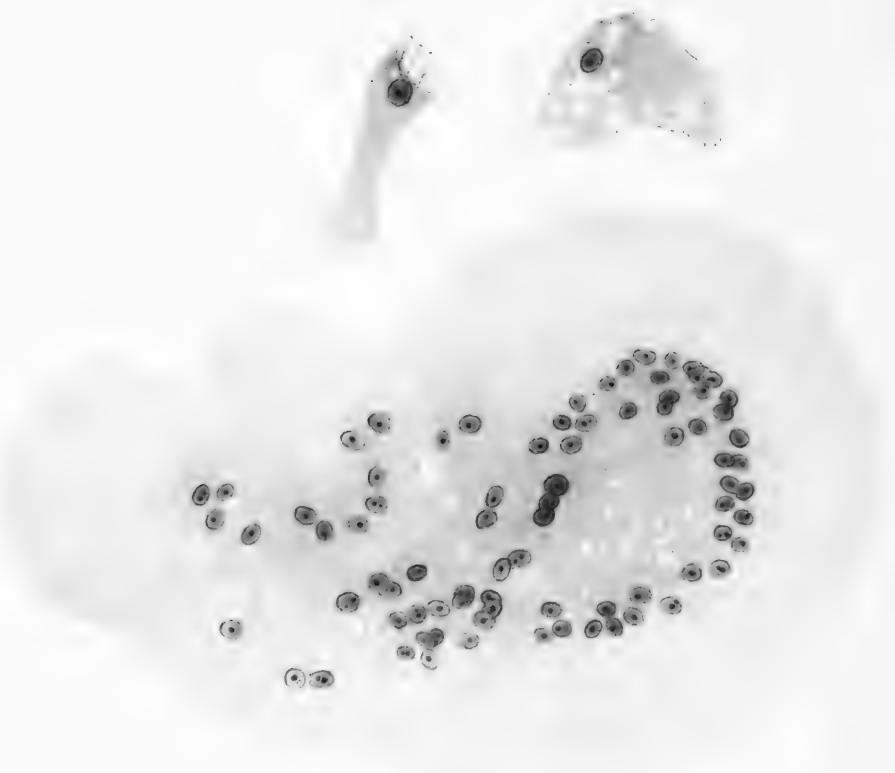

FIG. 7.

2. These giant cells are formed by the fusion of large mononuclear wandering cells, probably endothelial cells and pulp cells. Connective tissue cells do not take part in their formation.

3. The large giant cells sometimes seen spread out over the cover-glass in cultures of chick embryo spleen are probably foreign body giant cells, the cover-glass acting as the foreign body.

EXPLANATION OF PLATES.

PLATE 71.

FIG. 1. Photomicrograph of a two day culture of chick embryo spleen containing lycopodium spores. In the zone of wandering cells, cell masses are seen enclosing spores. The spores are stained with methylene blue; the tissue is unstained. Low power.

PLATE 72.

FIG. 2. Photomicrograph of a field in the preparation shown in figure 1. The spores and surrounding cells appear in greater detail. The characteristic architecture of the lycopodium is shown. High power.

FIG. 3. A group of wandering cells near the edge of the drop of plasma in a two day spleen culture. Leucocytes show pyknotic nuclei and ragged cytoplasm. The larger cells vary somewhat in their morphology.

PLATE 73.

FIG. 4. Section through a giant cell enclosing two lycopodium spores. The cell is from an eight day culture of chick embryo spleen. The spores are cut in median section.

FIG. 5. Section through the original piece of tissue containing a foreign body giant cell from a six day culture of chick embryo spleen. The lycopodium spore is cut tangentially.

FIG. 6. Giant cell formed about a piece of cotton fiber from a five day culture of chick embryo spleen.

PLATE 74.

FIG. 7. Large cover-glass giant cell and two mononuclear wandering cells, from an eight day spleen culture. The vacuoles in the cytoplasm represent fat droplets. The nuclei of the giant cell and of the small cells are identical in appearance.

ON THE PERMANENT LIFE OF TISSUES OUTSIDE OF THE ORGANISM.*

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PLATES 75 AND 76.

INTRODUCTION.

It is known that the life of a tissue cultivated *in vitro* is very short.[1] Generally, in three to fifteen days after the preparation of the cultures their growth becomes less rapid and eventually stops completely. The tissues then die and the cells disintegrate.

The purpose of the experiments described in this article was to determine the conditions under which the active life of a tissue outside of the organism could be prolonged indefinitely. It might be supposed that senility and death of the cultures, instead of being necessary, resulted merely from preventable occurrences; such as accumulation of catabolic substances and exhaustion of the medium. The suppression of these causes should bring about the regeneration of old cultures and prevent their death. It is even conceivable that the length of the life of a tissue outside of the organism could exceed greatly its normal duration in the body, because elemental death might be postponed indefinitely by a proper artificial nutrition.

In the following experiments I sought not a complete solution of the problem of prolonging indefinitely the life of tissues *in vitro*, but the facts that would show in which direction we should proceed in order to secure a complete solution, if such a solution be possible.

In September, 1911, I tried to increase the duration of the life of cultures of connective tissue.[2] The cultures were washed for

* Received for publication, March 15, 1912.

[1] Harrison, Harvey Lectures, Philadelphia and London, 1911–1912; *Jour. Exper. Zoöl.*, 1910, ix, 787; Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 387.

[2] Carrel, *Jour. Am. Med. Assn.*, 1911, lvii, 1611.

several minutes in Ringer's solution and were then placed in a new medium. As a consequence, the growth was observed to become more rapid. By repeated washings and passages the occurrence of senility was prevented, and the length of life was very much increased. Some cultures could be maintained in active life for fifty, fifty-five, and even for sixty days.[3] These results showed that the early death of tissues cultivated *in vitro* was preventable, and, therefore, that their permanent life was not impossible.

Subsequently, I developed other techniques by which it might be possible to obtain permanent life of tissues. The ideal method would have been to give to the cultures an artificial circulation by which they might have secured their nutrition and eliminated their waste products. It was attempted to place the tissues in a thin layer consisting of cellulose and coagulated plasma, and through this a slow stream of serum flowed. But it appeared soon that better results could be obtained by a simpler and more indirect method. Instead of establishing a circulation of the medium through the tissues, it was easier to have the cultures pass from medium to medium. The media usually employed consisted of Ringer's solution and hypotonic plasma. It was found that the resistance of a tissue placed in a non-nutritive, but preservative medium was very much stronger if the temperature was low. The cultures were, therefore, placed under conditions having two alternate phases: there was a phase of active life at 38° C. during which the tissue grew, used its medium, and was surrounded by its catabolic products; and a phase of latent life at 0° C. during which it was washed free of its products, to be subsequently placed in a new medium.

In February, 1912, this technique was modified and the phase of latent life in Ringer's solution in cold storage was replaced by a phase of active life in serum in the incubator. Instead of being interrupted, the life of the tissues was continuous and their growth more rapid.

METHODS.

The methods chiefly employed were three. They are as follows: *Technique I.*—The original piece of tissue and the surrounding

[3] Pozzi, *Bull. de l'Acad. de méd.*, 1912, lxvii, 26.

new cells were extirpated with a cataract knife from the fragment of coagulated plasma that contained them. They were washed for several minutes in Ringer's solution at the temperature of the laboratory, and were then placed in a new medium composed of one or two parts of distilled water and of three parts of normal plasma, to which a drop of embryonal or muscle extract was added. The washings and passage were made either before the appearance in the culture of the symptoms of senility, or when these were still very slight. The passage was repeated more or less frequently according to the rate of the growth and to the conditions of the cells.

Technique 2.—In the second method the washing of the cultures was more prolonged and was performed while the tissues were in cold storage. The life of the cultures was maintained under conditions in which two phases alternated: a phase of active life in a plasmatic medium in the incubator, and a phase of latent life in Ringer's solution in the refrigerator.

Technique 3.—In the third method the cultures were maintained in a condition of continuous life by alternating passages from plasma to serum at the temperature of the organism. The cultures were prepared in the following manner: A piece of silk veil about one centimeter square was placed on a cover glass and moistened with a drop of plasma. A fragment of tissue was deposited in the centre of the square and covered by a few drops of plasma. Afterwards the culture was sealed on a hollow slide, put in the incubator, and allowed to grow. After a few days the silk veil together with the coagulated plasma and tissue, was detached from the cover-glass with a knife. As the silk veil acted as a skeleton for the plasmatic jelly, the original fragment, and the new tissue cells, the culture could be handled easily without folding and retraction of the medium, and without deformation of the cells. The veil and the culture attached to it were placed in a tube containing homogenic serum, which was kept at incubator temperature. After a few days it was put again in plasma, and subsequently the two phases alternated regularly.

Experiments and Results.—A great many experiments were performed. In most of the cultures were used connective tissue of

blood vessels, heart, skin, muscles, peritoneum, and spleen, of fourteen to twenty day old chick fetuses. In other cultures were employed fragments of the Rous sarcoma, and in a few cases, tissues of adult dogs. Many cultures survived six to fourteen or fifteen passages in a condition of interrupted or continuous growth. A few cultures were transplanted seventeen, eighteen, and even twenty or more times. Some are still living and have reached the beginning of the third month of their life *in vitro*. It was possible, therefore, to study the morphological and dynamic characters of tissues cultivated for more than two months outside of the organism.

MORPHOLOGICAL CHARACTERS.

The appearance of the cultures grown by technique 1 was almost identical with that of the cultures grown by technique 2. The fragment of tissue was surrounded rapidly by cells spreading in a thin layer through the medium. After section of the plasma, the new tissue retracted around the original fragment forming an opaque crown. During the period of washing there was no modification of the culture, but as soon as the culture was put in a new medium in the incubator, elongated cells began to grow from the edges of the old plasma and to spread into the new medium. After a few passages the original fragment appeared diminished in size and surrounded by a dense envelope of a greyish tissue from which radiated a great many elongated cells, living and dead.

The original fragment was often encircled by several concentric rings, each representing a period of active life. Soon the rings were united in a homogeneous tissue. When they remained distinct, however, they might indicate the number of passages undergone by the culture, as the age of a tree can be read on a section of its trunk. Progressively the original fragment disappeared and was replaced by an amorphous yellowish substance. The form of the culture was also modified. At the beginning of the growth the culture appeared like a thin plate, but it grew progressively more and more like a flattened sphere in the center of which were accumulated a few foreign bodies and pieces of old plasma (figure 1), which were encysted by the living cells. After about forty days the

cultures of connective tissue generally took the typical appearance represented in the drawings (figures 2 and 3) of a culture fifty days old.

Cultivation of Connective Tissue (*Experiment 715*).—A short segment of the iliac vessels of a twenty-one day old chick fetus was extirpated on November 1, 1911, and placed in hypotonic chicken plasma and muscle extract. A large growth of connective tissue cells developed in the next few days.

On November 3 the plasma was cut and the culture was placed in Ringer's solution in cold storage from 9:52 A. M. to 10:35 A. M. Afterwards, the first passage into a new medium was made. A large growth resulted.

On November 6 the second passage was made after washing in Ringer's solution and keeping in cold storage from 9:55 A. M. to 10:58 A. M. At 2:30 P. M. fusiform cells had already invaded the new medium.

On November 9 the third passage was made after washing for six minutes in Ringer's solution, and on November 13 the fourth passage was made after washing in Ringer's solution and keeping in cold storage from 9:50 A. M. to 10:42 A. M. The iliac vessels were becoming progressively smaller, while a thick crown of cells in concentric lines surrounded the original fragment.

On November 16 the culture was washed in Ringer's solution and in cold storage from 9:20 A. M. to 10:12 A. M., and then placed in a medium composed of plasma and muscle extract. There was a large growth (fifth passage).

On November 20 the culture was washed in Ringer's solution in cold storage from 9 A. M. to 11:12 A. M., and then transferred to plasma and muscle extract (sixth passage).

On November 24 the culture was placed in a medium composed of hypotonic plasma and embryonic extract (seventh passage), after having been washed in cold storage and in Ringer's solution from 9 A. M. to 10:18 A. M.

On November 25 a large growth of cells was seen in the medium. The original fragment had almost completely disappeared, and the culture has a tendency to take a spherical form.

On November 28 the culture was placed in Ringer's solution and in cold storage from 9:30 A. M. to 12:02 P. M. and afterwards in hypotonic plasma and embryonal extract (eighth passage), and on November 29 a large growth was observed. The culture assumed the form of a flattened sphere, the center of which was clear and amorphous.

From December 2 to December 12 the culture underwent its ninth, tenth, eleventh, and twelfth passages. Before each passage it was washed for four or five minutes in Ringer's solution at the temperature of the laboratory.

On December 14 the culture was composed of a central amorphous part of a thick ring of new tissue, and of a peripheral crown of elongated cells radiating through the culture medium.

On December 15 the culture was placed into a new medium after having been washed for four minutes, and on December 16 a large growth through the new medium was seen. The rate of growth had markedly increased.

On December 18 the culture was washed for three minutes and underwent its fourteenth passage, in which a rapid growth of elongated tissue cells was observed.

On December 20 the culture was fixed in formalin and stained with hematoxylin. Two drawings of the stained specimen were made (figures 2 and 3), showing the amorphous center and its crown of actively growing tissue.

Even after seventy-five or eighty days, cultures of connective tissue made by the first two methods had about the same appearance, all of them resembling the later stages of the culture of the iliac vessel first described.

By the same method a few fragments of adult dog tissues, such as thyroid and periosteum, were cultivated, and several generations of connective tissue cells were obtained.

Frequently the cultures of Rous sarcoma died after a few days. The plasma became liquefied and the tissue disintegrated. Nevertheless, a small fragment of it could be kept in active life for forty-six days.

Culture of the Rous Sarcoma.—On January 24, 1912, a small piece of a Rous sarcoma was cultivated in hypotonic plasma. The growth was rapid and abundant. On January 26 the fragment was surrounded by an immense number of radiating fusiform cells and by a peripheral crown of round and polygonal cells. On January 26 and 29 the culture was washed for three or four minutes in Ringer's solution and put in a new medium. On January 30 fusiform cells only could be seen in the culture. On January 30 and on February 1, 3, 5, and 7 the culture underwent its third, fourth, fifth, sixth and seventh passages. The growth was not very active. On February 7 the culture was divided into two parts. One of these was washed and underwent its eighth passage on February 9. After the ninth and tenth passages on February 10 and 12, a microbian colony appeared in the medium. Nevertheless the active part of the culture could be extirpated without being contaminated and underwent its eleventh passage on February 15.

Afterwards the growth was small but aseptic. After the twelfth passage on February 17 the tissue produced a partial liquefaction of the medium with division or dissociation of the new tissue. Nevertheless, after the thirteenth passage on February 20 and the fourteenth passage on February 22 the tissue began to grow more abundantly and regularly without liquefaction of the medium. From February 24 to March 1 the culture underwent its fifteenth, sixteenth, seventeenth, and eighteenth passages. The growth was abundant and the culture increased very much in size. On March 5 a marked retraction of the plasma occurred. After the nineteenth and twentieth passages the growth became very small and death occurred without any apparent cause.

When the tissues were cultivated according to the third method the appearance of the cultures was different. Instead of being interrupted, the growth was continuous, and as there was no sectioning of the plasma, there was, as a consequence, no retraction.

Moreover, as the plasma was supported by its skeleton of silk, the culture could not take a spherical form, and the tissue grew in a thin layer; that is, under the best conditions for a tissue deprived of circulation. The plasmatic jelly was adherent to the silk veil, and the tissue imbedded in the plasma grew as in an ordinary culture. The cells could use the silk threads as a support, but they seldom did. The purpose of the silk was merely to give such a consistency to the medium that it would not fold over or become spherical during the passages in plasma and in serum.

Normal heart was cultivated according to this technique. Its appearance after a few passages was the same as after a few days. The size of the growth alone was modified.

By the same procedure many fragments of the Rous sarcoma were also cultivated. They could be kept in a condition of active and continuous development for more than fifteen days. But the medium was progressively overcrowded with dead cells and the growth of the tissue became more irregular and less abundant. The fragments could not adapt themselves to their new conditions of life and were less resistant than normal connective tissue.

The appearance of the cells growing in cultures of normal connective tissue often varied periodically. During the first few days of growth the fusiform cells were long and thin and contained clear cytoplasm. After four or five days they became larger and their cytoplasm was darker and filled with large fatty granulations. At the same time the rate of growth diminished. After being washed and transferred to a new medium, the appearance of the cells was again modified. They regained their original appearance and the rate of growth was accelerated. Instead of being periodical, the morphological modifications might be continuous. For instance, in the cultures of spleen the original fragment was surrounded during the first phases of active life by an envelope of ameboid cells. After a few days long chains of fusiform cells appeared around the fragment, while the ameboid cells died and disappeared. Ultimately the fusiform cells were replaced by small polygonal cells which spread around the fragment in a continuous layer. In the culture of the Rous sarcoma the round cells disap-

peared after a few days, while the elongated cells were still growing after forty days. In a culture of heart very large ameboid cells wandered out from the central fragment for more than two months. At the beginning of the third month of its life the culture was composed of elongated cells radiating through the old plasma. They were apparently the result of the development of the large ameboid cells.

DYNAMIC CHARACTERS.

The rate of growth could be appreciated by the rapidity with which the cells, after a passage, appeared and spread in the new medium. The nature of the medium, its osmotic tension, the way in which the plasma was cut, the amount of old plasma left around the cells, the form of the culture, and the frequence of the passages, often had a marked influence on the rate of the growth. The oscillations in the rate seemed generally to be the result of accidental causes, but it was very difficult to exclude the possibility that they might be due to causes inherent in the condition of tissues liberated from the control of its organism. Generally, after the first passage the cells appeared in the medium without a latent period. This was true even in the culture of periosteum and of other tissues of adult dogs. Two or three hours after a passage many elongated cells were observed growing out from the edges of the old plasma into the new medium, and they soon surrounded the central piece by a dense layer. The rapidity of their growth often diminished after a few passages, but afterwards increased progressively. The rate of growth of a culture forty or forty-five days old was more rapid than when it was ten or fifteen days old. It seemed that the older a culture was, the quicker it grew. The rate of growth of a fifty-four day old culture of connective tissue from the portal vein was more rapid than at the beginning of its life *in vitro*. The rate of growth of a culture of heart was greater at the beginning of the third month of its life outside of the organism than at any other period.

These accelerations of the growth were possibly functions of the age of the culture. But the number of the experiments was not large enough to exclude the possibility that they were due to other causes.

The size of the cultures underwent also many modifications. For long periods of time, the cultures increased in size, diminished, or were not modified.

The diminutions in size were due chiefly to accidental causes, such as destruction of cells during the handling of the cultures, folding of the plasma, and concentration of the medium, but especially to microbian infection.

In a few experiments marked increase in size was observed. The tissue of a culture of portal vein had been considerably reduced after infection, and for several generations it increased but slightly. Then the growth became more active and after the fiftieth day the volume of the tissue increased rapidly. In a culture of connective tissue which had remained very small for two months, a great acceleration of growth and a marked increase in size was observed after sixty days. In another instance a culture of heart became so large after sixty-two days that it had to be divided into several parts.

It is generally difficult to ascertain whether or not the mass of the tissue actually increases, because changes in the density of the tissue can be mistaken for a change in its mass. Nevertheless, the observation of a culture of a diluted suspension of cells demonstrated that a real increase in the volume of the tissue could take place.

Culture of Cells Suspended in Ringer's Solution.—On February 1, 1912, a nine day old chick embryo was cut into very small pieces, and to the pieces an equal volume of Ringer's solution was added. The suspension was centrifugalized for five minutes, and a drop of the supernatant fluid was then added to a culture medium. On February 2 many isolated cells were seen in the peripheral part of the medium. On February 5 the number of these cells had increased very much. The part of the plasma where they had grown was resected, washed, and put in a new medium. The culture underwent its second, third, fourth, and fifth passages on February 2, 10, 13, and 16. The cells had then increased so much in number that they formed a real tissue, and had the appearance of an ordinary culture. The life of these cells afterwards was like that of other cultures.

Since a few scattered cells could generate a culture similar to the culture of fragments of tissue, it is absolutely certain that the cells multiply and that they do not merely wander from the central piece.

Generally, the cultures remained very small in spite of their constant growth, and often they did not increase at all in size. It is probable that the limitation of growth is due to certain mechanical conditions. Cultures have a tendency to become spherical. The nutriment that reaches the center of such cultures is insufficient, but that which is supplied to the periphery is abundant. Hence, the cells at the periphery multiply ceaselessly. But as soon as cells are isolated from the medium by a sufficient thickness of new tissue, their nutrition probably becomes deficient and they disintegrate. While the culture increases constantly by its periphery, it diminishes in its center and therefore its volume does not increase.

A tissue deprived of circulation probably cannot grow in a nutritive medium beyond a certain volume which is a function of its permeability to the medium and consequently of the thickness of the culture. If the culture be spherical its volume will remain very small, since the nutrition of its center is insufficient. From a theoretical standpoint, on the other hand, a tissue growing in a very thin layer could increase indefinitely. It is for this reason that the tissues were cultivated on a silk veil, for the veil prevented the plasma of the culture from retracting and becoming spherical.

The attempt was also made to ascertain whether tissues after a long period of life *in vitro* could retain their normal functions. The experiments were carried out with fragments of chick fetus heart, which, as is well known, can grow and pulsate for several days when kept at a proper temperature in plasma. In December, 1911, it was observed that when the pulsations of a culture of heart stopped, they began again after the tissue had been washed and transplanted into a new medium. Then in January, 1912, the attempt was made to determine the interval during which the cardiac muscle would retain its property of contracting rhythmically. Three experiments gave positive and identical results. One of these will be described.

Cultivation of the Heart (*Experiment 720–1*).—On January 17, 1912, a small fragment of the heart of an eighteen day old chick fetus was cultivated in hypotonic plasma. The fragment pulsated regularly for a few days and grew extensively. After the first washing and passage on January 24 the culture grew again very extensively, but there were no rhythmical contractions. On

January 29 and February 1, 3, 6, 9, 12, 15, 17, 20, 24, and 28, the culture underwent eleven washings and passages. It became surrounded by fusiform cells and many dead cells. There were no pulsations. After the twelfth passage the culture did not grow at all. Then the tissue was dissected and the old plasma was completely extirpated. A small central fragment was removed, washed, and put in a new medium. On March 1 it was pulsating at a rate that varied between 60 and 84 per minute. On March 2 the pulsations were 104 at 41° C., and on March 3, 80 at 40° C., but on March 4 the pulsations were very weak and stopped altogether at 2 P. M. On March 5 the culture underwent its fourteenth passage, and the pulsations reappeared immediately. They became weak again on March 2. On March 8 the fifteenth passage was made. On March 9 the pulsations were again 80 to 82 per minute at 40° C., and on March 12 they were 60 per minute. They then became slower and weaker. After the sixteenth passage on March 12 the pulsations were irregular, and the fragment beat for a series of 3 to 4 pulsations, and then stopped for about 20 seconds. After the seventeenth passage on March 16 regular pulsations at 52 beats per minute reappeared, and the tissue grew abundantly. After the eighteenth passage on March 19 the pulsations were irregular.

It was thus demonstrated that a fragment of chicken heart could still pulsate rhythmically at the beginning of the third month of its life outside of the organism. It showed, consequently, that a fragment of tissue living *in vitro* could retain its normal function for a long time.

The maximum age that tissues living *in vitro* can reach is still undetermined. Many cultures have died after less than two months, but a few were very active at the beginning of the third month of their life outside of the organism. Generally, the tissues seemed, after a time, to adapt themselves to their new condition, and after the fourteenth or fifteenth passage, very few cultures died spontaneously. In the handling and changes required by the passages, the cultures were exposed to many accidents, chiefly to microbian infections. In case of a local infection the part of the culture that was not yet infected was resected with a cataract knife and placed in a new medium. Often the culture recovered and produced several generations of cells that were free from microbian infection. But when infection was generalized, the tissues always died rapidly. Many cultures died of sepsis. In the description that follows is an illustration of this kind of death.

Culture of Portal Vein (*Experiment 483*).—On September 27, 1911, a fragment of the portal vein of a chick fetus sixteen days old was cultivated in three

parts of chicken plasma and two parts of distilled water. The tissue grew very extensively. On October 2 the culture was divided into three parts. One was washed in Ringer's solution for five minutes and put in a new medium. On October 7 it had grown extensively and was again divided into two parts. One part was washed and transplanted into a new medium and a large growth followed. On October 9 and 12 the third and fourth washings and passages took place. On October 14 a very large growth of long, clear, slender fusiform cells was present. On October 16 the growth had stopped and the cells were large and contained very large fat granules. On October 16 the fifth washing and passage took place, and on October 17 the growth of fusiform cells, which were again clear with few granules, was very active. After the sixth passage on October 20 retraction of the medium occurred, and the growth of the tissue was slight. By October 22 the original fragment had diminished greatly in size. On October 23 and October 26 the culture underwent its seventh and eighth passages, and on October 27 the original fragment was no longer to be distinguished. The culture was composed of an amorphous central part around which a crown of active tissue had developed. The tissue was disposed in concentric circles and from its peripheral part elongated cells radiated through the culture medium (figure 1). After the ninth and tenth passages on October 30 and November 4, a great increase in the rate of the growth was observed, but after the eleventh passage on November 9 the culture assumed a spherical shape, but grew extensively nevertheless. On November 10, 13, 16, and 18, the twelfth, thirteenth, fourteenth, and fifteenth passages took place. The rate of growth was very rapid. One hour after the fifteenth passage, the fusiform cells had already invaded the new culture medium, but on November 19 a large microbian colony appeared on one side of the culture, and on November 20 the culture was invaded by a great many bacterial colonies. The death of the tissue occurred soon after.

Nearly all the cultures made in the latter part of 1911 died in the same manner after one to two months. By slight modifications of the technique, however, infection was almost completely eliminated in the experiments made in the beginning of 1912.

Of sixteen cultures of heart and blood-vessels made on January 17, 1912, five were still very active in March, 1912, and of the five active ones, two heart cultures previously described grew slowly, but pulsated, and another heart culture, which pulsated from time to time, produced a large growth of ameboid and fixed cells which covered an extensive area of the medium. In this instance after having been motionless for two months, the central part of the culture manifested strong rhythmical contractions on the sixty-fifth day of its life *in vitro*. Moreover, two cultures of connective tissue made on January 17 were growing actively at the beginning of April. The rate of growth and the increase in their volume became very much greater as they grew older.

SUMMARY AND CONCLUSION.

In two series of experiments made at the end of 1911 and at the beginning of 1912, new techniques were developed with the view of investigating the problem of prolonging indefinitely the life of tissues isolated from the organism. These techniques are far from perfect and will doubtless be modified in the future. But they have already permitted the establishment of new facts.

Fragments of connective tissue have been kept *in vitro* in a condition of active life for more than two months. As a few cultures are now eighty-five days old and are growing very actively, it is probable that, if no accident occurs, the life of these cultures will continue for a long time.

In some cases the rate of growth of the tissues increased in direct ratio to the age of the culture.

Fragments of heart pulsated rhythmically at the beginning of the third month of their life *in vitro*.

These facts show that experiments made with these or with more perfect techniques and followed over long periods of time may lead to the solution of the problem of permanent life of tissues *in vitro*, and give important information on the characters acquired by tissues liberated from the control of the organism from which they were derived.

EXPLANATION OF PLATES.

PLATE 75.

FIG. 1. A thirty day old culture of connective tissue. The center of the culture consists of debris of old plasma. Around it is a ring of concentric layers of very active new tissue.

PLATE 76.

FIG. 2. A fifty day old culture of connective tissue. Active tissue is encircling a piece of old plasma.

FIG. 3. Peripheral part of the same culture.

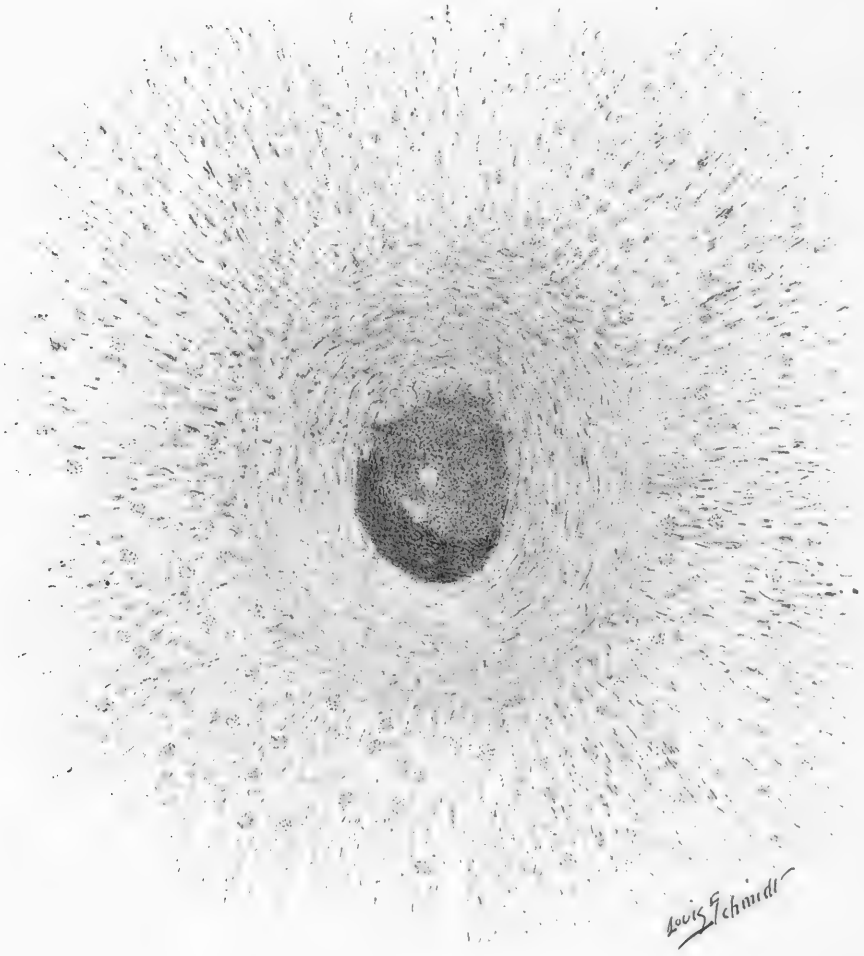

FIG. 1.

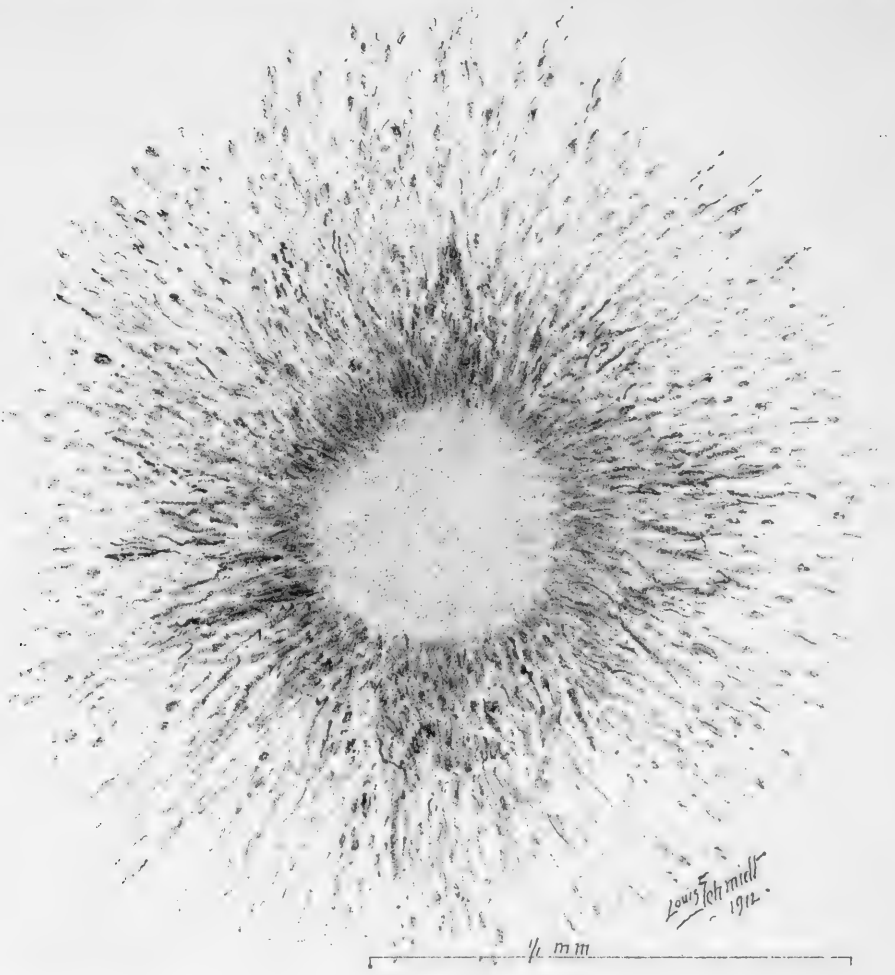

FIG. 2.

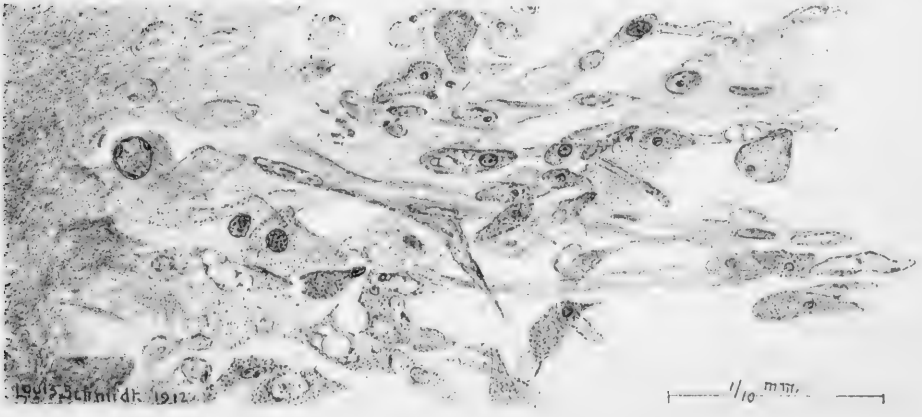

FIG. 3.

ON ALBUMINOLYSINS AND THEIR RELATION TO PRECIPITIN REACTIONS.*

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Much of the information that has been gained concerning antigen-antibody reactions, though primarily suggested by purely bacteriological investigations has been worked out in detail by the use of antigens of other than bacterial origin. Thus, much aid has been given to our comprehension of toxin-antitoxin phenomena by investigations on ricin and similar poisons; our knowledge of the amboceptor-complement reactions has been developed largely by studies in which red blood cells have been used as antigen; and our information regarding precipitin reactions is largely the result of studies carried on with unformed proteid antigens, such as animal sera, egg albumen, and others.

The great similarity in the response of animals to treatment with antigens from various sources has led to the recognition of certain broad principles of antibody formation which have often been used to justify theoretical opinions not amenable to direct experimentation. Thus recent theories of anaphylaxis and immunity have utilized the assumption of the existence of so-called albuminolysins, bodies analogous to the sensitizers, or amboceptors, known to react with cellular antigens, reacting in this case, however, with unformed proteids. Such albuminolysins form the keystone of Nicolle's (1) theories concerning anaphylaxis and immunity, and they recur in principle in the theories of Vaughan and Wheeler (2), Wolff-Eisner (3), and others.

Nevertheless, the conception that such albuminolytic bodies are completely analogous to the bacteriolytic or cytolytic sensitizers must be regarded at the present time as purely hypothetical.

* Received for publication, March 8, 1912.

In 1902, Gengou (4) showed that mixtures of milk, serum, egg-white, fibrinogen, animal sera, etc., with their specific antisera, gave rise not only to precipitates, but formed combinations which possessed the power of absorbing complement. He concluded from his experiments that the treatment of animals with unformed proteids resulted in the formation not only of precipitins, but also of specific sensitizers, or amboceptors, which, entirely analogous to the sensitizers for cellular elements, served to make possible the combination of alexin, or complement, with the antigen. In the light of subsequent research, it is doubtful whether this conclusion is justifiable.

A few years later (1905), Moreschi (5), working on anticomplements, confirmed Gengou's experimental results, showing that such complement fixation occurs even when minute quantities of antigens are added to their respective antisera, and Neisser and Sachs (6) soon applied this discovery practically to the differentiation of proteids in forensic cases showing its delicacy to be far greater than that of the precipitin tests. It was Gay (7), however, who first analyzed this phenomenon correctly and called attention to the fact that, in proteid-antiproteid mixtures made in sufficient concentration to give visible precipitates, it was the precipitates only that fixed complement, while all complement-binding properties were lacking in the supernatant liquids.[1]

The question now arises whether complement fixation by precipitates, as described above, is due to a mechanism entirely analogous to that governing a similar fixation by sensitized cellular elements. In the latter case, we know that there is a specific body, amboceptor, or sensitizer,—the latter term is preferable because it is non-committal,—through the mediation of which the action of complement upon the antigen is made possible. This sensitizer is, as far as we can tell at present, distinct from the precipitins. The fact that by the coöperation of this sensitizing body alterations may be wrought upon the antigen, resulting often in proteid cleavage and antigen disintegration, gives much importance to the question of whether analogous sensitizers are formed against the unformed proteids.

We may conceive the fixation of complement by precipitates to take place in two ways:

[1] This observation of Gay's is not necessarily in contradiction to the well established fact that complement may be fixed by proteid-antiproteid mixtures even when the quantities in the mixtures are so small that no visible precipitate is formed. For, by analogy with the conditions governing the mutual precipitation of inorganic colloids, it is not only conceivable but probable that actual visible precipitation occurs only when definite quantitative relationships are maintained. The fixing complex may, therefore, be formed without actual precipitation taking place (Arthur Müller, Allgemeine Chemie der Kolloide, Leipzig, 1907, p. 76).

1. There may be complete analogy between the response to immunization with formed cellular elements and the response to immunization with unformed proteids. If the analogy is complete, we may conceive (1) that the precipitin reaction is merely the visible effect of the combination of an antigen with the sensitizer, or (2) that in the formation of the precipitate, this lysin-like body is mechanically carried down, much as albumen may be carried down with globulin in the process of salting out with ammonium or magnesium sulphate.

2. The fixation of complement by precipitates may be a phenomenon *per se,* depending on a mechanism independent of that governing the reactions between formed antigen-amboceptor-complement complexes. In this case, we should have to look upon the albuminolysin as a separate body which may be present or absent independently of the presence or absence of the cell amboceptor, and following laws of its own.

The problem, sufficiently interesting in itself, has gained more than a theoretical significance by the recent work on anaphylatoxins, and it was in connection with studies on these bodies that the present observations were made.

In Friedberger's (8) earlier experiments, it appeared that an acutely toxic body could be produced by allowing complement to act upon washed serum precipitates. His subsequent work and that of others has shown that similar bodies resulted from the action of complement upon sensitized bacteria.

It would therefore be of great value if we could determine by actual experiment whether or not the body by the aid of which complement enters into relation with the serum antigen is identical with the sensitizer by the aid of which its combination with the cell or bacterial antigen is affected.

Such information is especially desirable, since the work of Neufeld and Dold (9) and others seems to show that anaphylatoxin production from bacteria does not go hand in hand with actual lysis, and a similar state of affairs was previously determined for red blood cells by Friedemann (10). The details of these investigations, however, cannot be appropriately considered in the present connection.

Related to, but not identical with the question with which the present paper deals are the investigations of Neufeld and Hühne (11), who noticed that the strongly bactericidal sera of two typhoid patients showed no complement fixation by the Bordet-Gengou technique, and concluded that the bacteriolytic antibodies and the complement-fixing "Bordetsche Antikörper" are quite distinct.

Similar observations have been made by Haendel (12) and by Toyosumi (13), and more recently Torrey (14), working with gonococcus immune sera,

noticed that antigonococcic serum may contain fixative substances which bind complement by the Bordet-Gengou method, and yet be devoid of bactericidal antibodies.

In the work recorded in the following protocols, the writer has attempted to throw some light on the question discussed above, by carefully comparing complement fixation as it occurs in serum and antiserum complexes with that which occurs in the reactions between bacterial antigen and antisera, since in the latter phenomena we know definitely of the existence both of precipitins and of lytic sensitizers in the immune sera.

I.

The work was begun by again going over the data concerning complement fixation by serum-antiserum reactions already accurately studied by Moreschi, Gay, Muir and Martin (15), Neisser and Sachs, and others.

The purpose of the first experiments was to determine, in sheep serum-antisheep serum mixtures, the fixation of complement by washed precipitates and by the supernatant fluids over such precipitates.

1. Sheep Serum plus Antisheep Rabbit Serum.—Rabbit 80 received three injections of 2, 5, and 10 c.c. of sheep serum at six day intervals, and was bled on the eighth day after the last injection. Precipitin reactions were carried out with 0.2 c.c. of antisheep serum and diminishing quantities of antigen in 4 c.c. volumes. After separating the precipitates from the supernatant liquid by centrifugalization, they were washed twice in salt solution.

Precipitate and supernatant liquid were then separately tested for complement fixation by adding the indicated quantities of complement, leaving them at 40° C. for two hours, and then adding washed red blood cells saturated with amboceptor.

The amount of precipitate formed and the result of the complement fixation tests with this precipitate are shown in the following table.

In the controls, 0.05 c.c. of complement hemolyzed sensitized red cells completely in 20 minutes. The supernatant liquid in no case fixed 0.05 c.c. of complement.

2. Sheep Serum plus Antisheep Rabbit Serum.—Precipitin reactions were carried out with 0.1 c.c. of antisheep serum from rabbit 65, and with diminishing quantities of antigen in 2 c.c. volumes. The precipitates were washed twice with salt after centrifugalizing and pipetting off the supernatant liquids. The amount of precipitate formed in each case is as follows:

		Precipitate
1.	0.1 c.c. antisheep serum + 2 c.c. sheep serum 1:50	+++++
2.	0.1 c.c. antisheep serum + 2 c.c. sheep serum 1:100	+++++
3.	0.1 c.c. antisheep serum + 2 c.c. sheep serum 1:200	++++
4.	0.1 c.c. antisheep serum + 2 c.c. sheep serum 1:500	++++
5.	0.1 c.c. antisheep serum + 2 c.c. sheep serum 1:1,000	+++
6.	0.1 c.c. antisheep serum + 2 c.c. sheep serum 1:3,000	++
7.	0.1 c.c. antisheep serum + 2 c.c. sheep serum 1:5,000	+

	Precipitate.	0.2 c.c. complement.	0.05 c.c. complement.
1. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:20	+++++	No hemolysis	No hemolysis
2. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:40	+++++	No hemolysis	No hemolysis
3. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:100	+++++	No hemolysis	No hemolysis
4. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:200	+++++	No hemolysis	No hemolysis
5. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:500	++++	No hemolysis	No hemolysis
6. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:1,000	+++	No hemolysis	No hemolysis
7. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:1,500	++	Very slight hemolysis	No hemolysis
8. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:2,000	++	Slight hemolysis	No hemolysis
9. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:5,000	+	Distinct hemolysis	No hemolysis
10. 0.2 c.c. antisheep serum + 4 c.c. sheep serum 1:10,000	Very faint turbidity	Almost complete hemolysis	Slight hemolysis

With the supernatant fluid separated by centrifugalization and with the washed precipitates, the complement fixation tests were made. In each case, the fixation was allowed to go on for two hours at 40° C., and then one cubic centimeter of sheep corpuscles saturated with amboceptor was added. The saturation was effected by adding an excess of amboceptor to a 5 per cent. emulsion of washed sheep corpuscles. Then, after keeping the mixture in the incubator for one hour, the corpuscles were washed. On testing the supernatant liquid from such mixtures with unsensitized corpuscles and 0.1 c.c. of complement, an excess of amboceptor was shown to be present. The three similar series of complement fixation tests follow.

SERIES A.

Washed precipitate from		Hemolysis in 5 min.	10 min.	30 min.	Next morning
1. Antigen dilution 1:50	Kept in 2 c.c. salt solution + 0.05 c.c. complement for 2 hrs. at 40° C., then 1 c.c. of sensitized blood cells was added.	None	None	None	None
2. Antigen dilution 1:100		None	None	None	None
3. Antigen dilution 1:500		None	None	None	None
4. Antigen dilution 1:1,000		None	None	None	None
5. Antigen dilution 1:3,000		None	None	None	Very slight
6. Antigen dilution 1:5,000		None	None	Very slight	Slight

Supernatant liquid from		Hemolysis in 5 min.	10 min.	30 min.	Next morning.
1. 2 c.c. of antigen dilution 1:50	+ 0.05 c.c. complement	None	None	Distinct	Complete
2. 2 c.c. of antigen dilution 1:100	for 2 hrs.	None	None	Complete	Complete
3. 2 c.c. of antigen dilution 1:500	at 40° C.,	Slight	Slight	Complete	Complete
4. 2 c.c. of antigen dilution 1:1,000	then sensitized	Distinct	Distinct	Complete	Complete
5. 2 c.c. of antigen dilution 1:3,000	red blood cells	Distinct	Distinct	Complete	Complete
6. 2 c.c. of antigen dilution 1:5,000	were added.	Distinct	Distinct	Complete	Complete

Hemolysis in all these tubes was complete in forty minutes.

Control with 0.05 c.c. of complement and 1 c.c. of washed sensitized corpuscles showed complete hemolysis in five minutes.

SERIES B.

Washed precipitate from		Hemolysis in 5 min.	10 min.	30 min.	Next morning.
1. Antigen dilution 1:50	+ 0.15 c.c. complement for 2 hours at 40° C., then sensitized red corpuscles were added.	None	None	None	None
2. Antigen dilution 1:100		None	None	None	None
3. Antigen dilution 1:500		None	None	None	None
4. Antigen dilution 1:1,000		None	None	Slight	Almost complete
5. Antigen dilution 1:5,000		None	Very slight	Distinct	Complete

Supernatant fluid of		Hemolysis in 5 min.	10 min.	30 min.	Next morning.
1. Antigen dilution 1:50	+ 0.15 c.c. complement for 2 hours at 40° C., then sensitized red blood cells were added.	Very slight	Marked	Complete	–
2. Antigen dilution 1:100		Very slight	Almost complete	Complete	–
3. Antigen dilution 1:500		Distinct	Complete	Complete	–
4. Antigen dilution 1:1,000		Distinct	Complete	Complete	–
5. Antigen dilution 1:3,000		Distinct	Complete	Complete	–
6. Antigen dilution 1:5,000		Distinct	Complete	Complete	–

Control with 0.15 c.c. of complement showed complete hemolysis in five minutes.

It was furthermore determined by experiments too simple to require tabulation that none of the complement-fixing elements could be washed out of the precipitates with salt solution.

SERIES C.

Washed precipitate from		Hemolysis in 5 min.	10 min.	30 min.	Next morning.
1. Antigen dilution 1:50	+ 0.03 c.c. complement for 2 hours at 40° C., then sensitized blood cells were added.	None	None	None	None
2. Antigen dilution 1:100		Very slight	Marked	Marked	Complete
3. Antigen dilution 1:500		Slight	Almost complete	Complete	—
4. Antigen dilution 1:1,000		Distinct	Complete	Complete	—
5. Antigen dilution 1:3,000		Distinct	Complete	Complete	—
6. Antigen dilution 1:5,000		Distinct	Complete	Complete	—

Supernatant fluid from		
1. Antigen dilution 1:50 2. Antigen dilution 1:100 3. Antigen dilution 1:500 4. Antigen dilution 1:1,000 5. Antigen dilution 1:3,000 6. Antigen dilution 1:5,000	+ 0.02 c.c. of complement for 2 hours at 40° C., then sensitized cells were added	Tubes 1, 2, and 3 were incompletely hemolyzed on the next day. Tubes 4, 5, and 6 were completely hemolyzed after 1 hour.

Control with 0.02 c.c. of complement showed complete hemolysis in forty-five minutes. The supernatant fluid from series C was treated with less complement, since in series B with 0.15 c.c. of complement no inhibition had been evident.

From the preceding experiments and others like them, it was evident that, as Gay, Moreschi, and others have shown, washed precipitates from serum-antiserum mixtures possess the property of fixing complement, and that the degree of such fixation is roughly proportionate to the bulk of the precipitate.

Except for occasional slight complement fixation by the supernatant fluids of the tubes in which the largest quantities of antigen had been used, the fixing power resided entirely in the precipitates.

It is not possible to state with certainty the cause of the occasional slight fixation by these supernatant fluids. However, since Gay has shown that precipitate redissolved by an excess of antigen does not lose its power of fixing complement, it may well be that the excess of antigen in the tubes in question accounted for this phenomenon.

II.

It was desirable next to carry out with bacterial antigen and antibacterial serum experiments analogous to the preceding, in order that a comparison of the conditions in such cases with those prevailing in the serum-antiserum experiments might be made.

In all protocols given below, the antigen used consisted of an alkaline broth filtrate of typhoid cultures. The cultures in flasks were allowed to stand in the incubator for eight weeks, and were then filtered through Berkefeld candles. The antisera used were obtained from rabbits that had received five or six intravenous injections of dead typhoid bacilli and had been bled seven or eight days after the last injection. Throughout the experiments the same strain of typhoid bacilli (*Bacillus typhosus* 65) was used both for immunization and antigen production.

The preliminary experiments were intended merely to determine the relative powers of complement fixation possessed by precipitates and supernatant fluids resulting from mixtures of bacterial antigen and antiserum.

Experiment 1. Typhoid Antigen and Antityphoid Serum.—A series of tubes was prepared each containing 2 c.c. of the typhoid filtrate and 0.1 c.c. of inactivated serum of rabbit 76, bled one week after receiving the sixth injection of typhoid bacilli.

In all these tubes after fifteen minutes at 40° C. there appeared a distinct turbidity which grew deeper during the next two hours. The tubes were then set away in the refrigerator until the next morning. At that time centrifugalization at high speed was used to separate a clear supernatant liquid from a very slight white granular precipitate. This latter was washed twice in salt solution and reëmulsified in 2 c.c. of physiological salt solution.

The following complement fixation tests were then carried out:

A.

		Hemolysis in	
		1 hour.	Next morning.
1. Washed precipitate +0.05 c.c. complement	Kept at 40° C. for 2 hours, then 1 c.c. of sensitized red blood cells was added.	None	Very slight
2. Washed precipitate +0.1 c.c. complement		Distinct	Complete
3. Washed precipitate +0.15 c.c. complement		Complete	—
4. Washed precipitate +0.2 c.c. complement		Complete	—
5. Washed precipitate +0.3 c.c. complement		Complete	—

A large number of experiments like the preceding, but varying somewhat in quantitative relationships, all brought out the point that the experiments with bacterial antigen and antiserum differed in an important way from those with serum-antiserum, for in the former, distinct complement-fixing properties were manifest not only in the precipitates, but also in the supernatant liquids.

B.

		Hemolysis in	
		1 hour.	Next morning.
1. Supernatant liquid (from A, 1.) + 0.05 c.c. complement	Kept at 40° C. for 2 hours, then 1 c.c. of sensitized red blood cells was added.	None	None
2. Supernatant liquid (from A, 2.) + 0.1 c.c. complement		None	None
3. Supernatant liquid (from A, 3.) + 0.15 c.c. complement		Very slight	Complete
4. Supernatant liquid (from A, 4.) + 0.2 c.c. complement		Complete	Complete
5. Supernatant liquid (from A, 5.) + 0.3 c.c. complement		Complete	Complete

Controls showed that no complement fixation took place with the rabbit serum alone or with antigen alone.

Since, however, the total quantity of complement absorbed by both precipitate and supernatant liquid in these experiments was very small, it seemed to the writer not unlikely that there might be present in the supernatant fluid a considerable excess of free amboceptor. In order to ascertain this definitely, a number of experiments were carried out. The following is a typical one.

Experiment 2.—To determine the presence of an excess of amboceptor in the supernatant fluids of the bacterial precipitin tests, 0.1 c.c. of antityphoid serum of rabbit 91 was added to 2 c.c. of antigen filtrate in each of a number of tubes. These tubes were kept at 40° C. for two hours, by which time a distinct turbidity had appeared. They were then placed in the ice chest over night, and after this the mixtures were centrifugalized, the supernatant liquid decanted, and the precipitate, which was very slight, was washed. The following tubes were then prepared:

			Hemolysis after 2 hours.
1. Washed precipitate	+ 0.1 c.c. complement	After 2 hours at 40° C. sensitized cells were added.	None
2. Washed precipitate	+ 0.2 c.c. complement		Very slight

3. Supernatant fluid	+ 0.1 c.c. complement	After 2 hours at 40° C. sensitized cells were added.	None
4. Supernatant fluid	+ 0.2 c.c. complement		Very slight
5. Supernatant fluid	+ 0.3 c.c. complement		Marked

					Hemolysis.
6. Supernatant fluid	+ 2 c.c. antigen	Kept for 1 hour at 40° C.	+ 0.3 c.c. complement	2 hours at 40° C., then sensitized cells were added.	None
7. Supernatant fluid	+ 2 c.c. antigen		+ 0.4 c.c. complement		None
8. Supernatant fluid	+ 2 c.c. antigen		+ 0.5 c.c. complement		Very slight
9. Supernatant fluid	+ 2 c.c. antigen		+ 0.6 c.c. complement		Complete

In tubes 6, 7, 8, and 9 of the protocol given above, it is evident that the addition of more antigen to the supernatant liquids from

mixtures of 0.1 of a cubic centimeter of serum and two cubic centimeters of antigen from which the precipitates had been removed, resulted in a considerable enhancement of the fixing power. Similar results were obtained when instead of antigen filtrate, washed bacteria were added to the tubes of supernatant fluid. Since, of course, the antigen alone did not fix complement, the deduction must be made that an excess of amboceptor in the original supernatant fluid accounted for its limited fixing power.

In tubes in which the addition of more antigen to the supernatant fluid gave rise to a second faint precipitate, a separate determination of the complement-binding properties of this second precipitate (it never fixed more than 0.05 of a cubic centimeter of complement) showed that such a precipitate did not account for the discrepancy.

III.

Since, in the case of the bacterial precipitin tests, it had been established that not only the precipitates, but also the supernatant fluids possessed complement-binding properties, it seemed to the writer that it might be possible to determine whether or not the complement fixation of the precipitates and that of the supernatant liquids depended upon one and the same mechanism. For, if this were the case, the removal of the precipitate should leave behind in the supernatant liquid less of the complement-binding body than was present in the original amount of serum used. In other words, in the presence of bacteria washed until the washings showed no trace of dissolved antigen, the supernatant fluid should possess less complement-fixing power than the corresponding amount of the original serum.[2]

On the other hand, if the complement fixation by the precipitate is due to a mechanism having no relation to the one in the supernatant fluid, the latter, in the presence of washed bacteria, should fix as much complement as a corresponding amount of serum would fix under similar conditions.

The experimental approach to this problem required much repetition, and several methods of procedure were employed. These will be described separately.

[2] This was determined by adding antiserum and complement to the washings and testing for fixation with sensitized cells in the usual way.

Experiment 1.—In these experiments were used inactivated antityphoid serum 77a, antigen filtrate from six weeks' broth cultures of *B. typhosus* 65, fresh guinea pig serum for complement, and twenty-four hour agar slant cultures of *B. typhosus* 65 for the bacterial emulsions.

By preliminary experiments, a determination was made of the amount of emulsion of washed bacteria necessary to saturate completely the complement-fixing bodies contained in 0.05 c.c. of serum 77a. This was done by determining the total quantity of complement absorbed in several series of tubes.

In one series each tube contained 0.05 c.c. of serum 77a and washed bacteria from 2 agar slants in 2 c.c. of salt solution. These tubes were allowed to stand at 40° C. for 1 hour; then complement ranging from 0.5 c.c. upward was added to each tube and, after 2 hours at 40° C., sensitized red cells were added. In another series, the tubes contained 0.05 c.c. of serum 77a and washed bacteria from 3 slants in 2 c.c. of salt solution. Complement was again added in the same way, and was followed by sensitized cells.

It was found that, when 0.05 c.c. of the serum was used, addition of bacteria in excess of two slants no longer enhanced the complement-fixing properties. In the presence of this quantity of washed bacteria,[3] 0.05 c.c. of serum 77a caused complete fixation of 0.6 c.c. of complement.

These preliminary determinations having been made, the actual experiment was carried out as follows:

A series of tubes was prepared, each of which contained 2 c.c. of antigen filtrate, and 0.1 c.c. of serum 77a. Turbidity appeared in five minutes, and was deeper in one hour. After twelve hours in the refrigerator, a slight, granular precipitate had settled down, which was separated by centrifugalization. This precipitate was washed with 2 c.c. of salt solution, and the washings were added to the supernatant fluid. The latter then represented 0.1 c.c. of the original serum in a 4 c.c. volume.

Tubes were then prepared as follows:

SERIES 1.

1. 2 c.c. supernatant fluid (representing 0.05 c.c. serum) + washed bacteria	Kept for 1 hour at 40° C.
2. 2 c.c. supernatant fluid (representing 0.05 c.c. serum) + washed bacteria	
3. 2 c.c. supernatant fluid (representing 0.05 c.c. serum) + washed bacteria	
4. 2 c.c. supernatant fluid (representing 0.05 c.c. serum) + washed bacteria	
5. 2 c.c. supernatant fluid (representing 0.05 c.c. serum) + washed bacteria	

To these tubes complement was then added as follows:

[3] The darker color which the blood emulsion usually takes in the presence of typhoid bacilli, does not interfere with accurate readings. However, the surprising fact that washed typhoid bacilli will by themselves occasionally fix small amounts of complement, must be controlled. This fixation never exceeded 0.05 or 0.0025 c.c. of complement, but is mentioned here because it was on one or two occasions unmistakable and is quite at variance with our usual conceptions.

		Hemolysis	
		After 1 hour.	Next morning.
1. As above + 0.6 c.c. complement	Kept for 2 hours at 40° C., then sensitized red cells were added.	None	None
2. As above + 0.65 c.c. complement		Slight	Distinct
3. As above + 0.7 c.c. complement		Slight	Almost complete
4. As above + 0.75 c.c. complement		Marked	Complete
5. As above + 0.8 c.c. complement		Almost complete	Complete

SERIES II.

1. 0.05 c.c. serum 77a in 2 c.c. salt solution + washed bacteria
2. 0.05 c.c. serum 77a in 2 c.c. salt solution + washed bacteria
3. 0.05 c.c. serum 77a in 2 c.c. salt solution + washed bacteria
4. 0.05 c.c. serum 77a in 2 c.c. salt solution + washed bacteria
5. 0.05 c.c. serum 77a in 2 c.c. salt solution + washed bacteria

} 1 hour at 40° C.

Complement was then added as follows:

		Hemolysis	
		After 1 hour.	Next morning.
1. As above + 0.6 c.c. complement	Kept for 2 hours at 40° C., then sensitized red cells were added.	None	None
2. As above + 0.65 c.c. complement		None	Slight
3. As above + 0.7 c.c. complement		Very slight	Marked
4. As above + 0.75 c.c. complement		Marked	Complete
5. As above + 0.8 c.c. complement		Complete	Complete

A comparison between series I and II shows that the complement bound by the two series is almost identical, the difference being well below 0.05 c.c. of complement.

The precipitate from the same experiments showed complement fixation as follows:

	Hemolysis	
	After 1 hour.	Next morning.
1. Washed precipitate from 0.1 c.c. of original serum + 0.05 c.c. of complement	None	None
2. Washed precipitate from 0.1 c.c. of original serum + 0.1 c.c. of complement	None	None
3. Washed precipitate from 0.1 c.c. of original serum + 0.15 c.c. of complement	None	Very slight
4. Washed precipitate from 0.1 c.c. of original serum + 0.2 c.c. of complement	Distinct	Complete

By dividing these results in half, we see that the precipitate from 0.05 c.c. of the original serum fixes more complement than that indicated by the slight difference between series I and II.

Nevertheless, because of the experimental difficulties and the slight quantitative differences, the above results, which were selected as a fair example from a large number of experiments, were not taken as final.

It was deemed desirable to devise a method by which more exact uniformity could be attained. For this reason a number of experiments were carried out by the following methods:

Experiment 2.—The same materials were used as in experiment 1. In two centrifuge tubes the following mixtures were made:

(A) 10 c.c. of antigen filtrate + 0.5 c.c. of serum 77a.

(B) 10 c.c. of salt solution + 0.5 c.c. of serum 77a.

In tube A, a turbidity appeared which was allowed to deepen for one hour at 40° C., after which the tubes were put into the refrigerator until the next morning. The precipitate was then thrown down by the centrifuge and the supernatant fluid pipetted away. This supernatant fluid then represented 0.05 c.c. of serum 77a in each 1 c.c. volume, or 0.025 c.c. of serum in each 0.5 c.c. volume.

SERIES 1.

In each of a series of tubes, 0.5 c.c. of the supernatant fluid was added to an emulsion of one agar slant of washed bacteria. These mixtures were kept at 40° C. for one hour. To these tubes were then added varying quantities of complement, and fixation was determined as follows:

		Hemolysis	
		After 1 hour.	Next morning.
1. As above + 0.2 c.c. of complement	Kept for 2 hours at 40° C., then sensitized red cells were added.	None	None
2. As above + 0.25 c.c. of complement		None	None
3. As above + 0.3 c.c. of complement		None	None
4. As above + 0.35 c.c. of complement		Very slight	Slight
5. As above + 0.4 c.c. of complement		Slight	Distinct

The results in experiment 2 differ from those in experiment 1 on account of the fact that, although other ingredients were the same, a different lot of complement was used.

SERIES II.

With the contents of tube B, a similar series of complement fixation tests were carried out. In this series, 0.5 c.c. of the serum salt solution mixture was mixed with an emulsion of washed bacteria and left for one hour at 40° C.

In the example given above, series I (containing the antigen filtrate) seems to have exerted slightly greater fixing powers than series II; but in other experiments, series II at times seemed to have fixed more complement than series I. It was necessary therefore to take into consideration all fluctuations, these being due to apparently unavoidable experimental errors. When this was done, the

		Hemolysis	
		After 1 hour.	Next morning.
1. As above +0.2 c.c. of complement	Kept for 2 hours at 40° C., then sensitized red cells were added.	None	None
2. As above +0.25 c.c. of complement		None	None
3. As above +0.3 c.c. of complement		None	Slight
4. As above +0.35 c.c. of complement		Very slight	Distinct
5. As above +0.4 c.c. of complement		Marked	Almost complete

results pointed definitely to the conclusion that the mechanism by which complement is fixed to the washed bacteria is not impaired or diminished by removal of the precipitate, and is, therefore, independent and distinct from that which fixes complement to the precipitate.

Although the above experiments, in their frequent repetitions, appeared almost conclusive, nevertheless it seemed wise to gain still greater assurance by another method of approach. For this purpose experiments were carried out of which the following is typical.

Experiment 3.—A. In a bacterial precipitin reaction, 0.5 c.c. of serum 84 was added to 9.5 c.c. of typhoid antigen filtrate, as above. After 10 minutes marked turbidity had appeared, and this became somewhat deeper after two hours in the incubator. *B.* In another test tube, 0.5 c.c. of the same serum was added to 9.5 c.c. of salt solution, and both tubes were kept in the refrigerator over night.

On the following day, the granular precipitate was removed from tube A by centrifugation. Tube A then contained in 10 c.c. the constituents of 0.5 c.c. of serum 84 minus the bodies which had gone into the precipitate, and tube B contained in 10 c.c. the unchanged constituents of 0.5 c.c. of serum 84.

Parallel sets of dilutions of A and B were then carefully made as follows:

1. 1 c.c. representing 0.05 c.c. of original serum.
2. 1 c.c. representing 0.01 c.c. of original serum.
3. 1 c.c. representing 0.005 c.c. of original serum.
4. 1 c.c. representing 0.0025 c.c. of original serum.
5. 1 c.c. representing 0.0001 c.c. of original serum.
6. 1 c.c. representing 0.00005 c.c. of original serum.
7. 1 c.c. representing 0.00001 c.c. of original serum.

To each tube was added 1 c.c. of typhoid antigen filtrate and 0.05 c.c. of complement, for greater accuracy in measuring the latter being added in a dilution of 1:10. The two series thus prepared were kept at 40° C. for two hours. At the end of this time, 1 c.c. of washed red cells saturated with amboceptor was added to each tube. The series were then carefully observed at intervals of ten minutes to determine whether there was exact parallelism in the speed of hemolysis, and final readings were taken twelve hours later. Complete parallelism between the two series could be established in a number of experiments, showing that the amboceptor, or sensitizer contents of the serum in tube A had not been altered by the removal of the precipitate, thus corroborating the results of experiment 2.

The precipitates from tube A in each of three experiments caused complete fixation of 0.5 to 0.6 c.c. of complement.

IV.

The experiments recorded in the preceding protocols have shown that the removal of the precipitate from reaction mixtures of typhoid antigen and antityphoid serum did not quantitatively detract from those bodies by means of which complement is fixed to the whole bacteria. In the method of experimentation employed, however, one important question was left unclear. If it is granted that the body which fixed complement in the precipitate, and the amboceptor, or sensitizer, which fixed it to the washed whole bacterial cell, are not identical, it is still important to determine whether, in the absence of dissolved bacterial proteid, the fixing body of the precipitin reaction can be bound, together with this sensitizer, to the bacterial cell. In other words, when inactivated immune serum is added directly to washed whole bacteria, do the bacterial cells bind both lytic amboceptor, or sensitizer, and that body which, in experiments otherwise conducted, binds complement in precipitates? To solve this question, a number of experiments were carried out as follows:

Experiment 1.—Antityphoid serum 84, inactivated at 56° C., *B. typhosus* 65, and typhoid antigen filtrate were employed.

Of the immune serum, 0.1 c.c. was added in a centrifuge tube to typhoid bacilli from five slants, washed five times in salt solution and emulsified in 1 c.c. of salt solution. The mixture was allowed to stand for three hours at room temperature, and then over night in the refrigerator at a temperature between 2° and 5° C. At the end of this time the mixture was centrifugalized until the supernatant fluid was entirely clear.

A complement fixation test now showed that 0.5 c.c. of complement was completely fixed by the bacteria from the above mixture, indicating, as was to be expected, that a large amount of amboceptor had become attached to them.

To the supernatant fluid was then added 2 c.c. of the typhoid antigen filtrate, and controls were made as follows:

1. Experiment.	Supernatant fluid from above	1.1 c.c.
	Typhoid antigen filtrate	2.0 c.c.
2. Control.	Antiserum 84	0.1 c.c.
	Salt solution	1.0 c.c.
	Typhoid antigen filtrate	2.0 c.c.
3. Control.	The same as 2.	

After ten minutes at 40° C., all these tubes had become turbid, showing that in tube 1 the previous treatment with washed whole bacteria had not deprived the 0.1 c.c. of the serum of the precipitin factor. It was noticeable, moreover, that the precipitate was slightly more rapid in forming and heavier in final result than that of the control tubes. This peculiarity, reappearing to a greater or lesser degree in all of four experiments, will be more fully studied in another series of experiments.

The precipitate from tube 1 as well as those from the control tubes 2 and 3, completely fixed 0.75 c.c. of complement, showing that in the point essential to our present investigation, these precipitates were identical.

We have, however, not yet made experiments in which we have succeeded in completely removing all cell amboceptor from the antiserum by treatment with whole bacteria. This is due to the fact that we did not wish to risk the possible solution of typhoid proteid by carrying out the preliminary exposure of serum to bacteria at 40° C. This, however, will be done later for a more complete study of this feature of the work, but we do not consider it essential for elucidation of the point at present under discussion.

These experiments show satisfactorily that the precipitin body, being in no degree removed from serum by exposure to whole bacteria, is not identical with the amboceptor (or sensitizer body) which reacts with the bacterial cell.

V.

SUMMARY AND CONCLUSIONS.

The writer believes that the experiments above recorded have demonstrated the following points:

1. As has been previously shown by other workers, complement is fixed by the precipitate formed in the reaction between unformed proteid and its homologous antiserum, and it is the precipitate only which fixes, the supernatant fluid being devoid of fixing power except to a slight degree in tubes very rich in antigen. This latter fixation is probably due to a redissolving of the precipitin-precipitinogen complex which, as Gay has shown, does not lose its fixing

power on resolution. The fixation of complement by mixtures in which antigen is so slight in amount that no precipitate is formed may be explained by analogy with colloidal reactions in which combination of colloids takes place without precipitation unless definite quantitative relations are maintained.

2. In contrast to the results above described, precipitating mixtures of bacterial antigen and antiserum show complement fixation both by the precipitate and by the supernatant fluid.

3. The complement fixation by the supernatant fluid of such bacterial precipitin reactions, with the precipitate removed, is, in the presence of washed bacteria, equal to that exerted by the same amount of the original serum. The complement fixation of the precipitates, here, as in serum-antiserum tests, is proportionate to the bulk of the precipitate.

4. The precipitin body is not removed from a bacterial antiserum by treatment with washed whole bacteria, whereas the cell sensitizer or amboceptor is removed by such treatment.

The writer believes that the work recorded above justifies Gengou's conclusion that an albuminolytic sensitizer is formed in response to immunization with unformed proteids. This sensitizer, or amboceptor, is, however, distinct and independent of the sensitizer that reacts specifically with either bacterial or other cells.

It would appear that immunization with unformed proteids calls forth only the formation of these albuminolysins, while immunization with cells, in our case the typhoid bacillus, calls forth both the cytolytic and the albuminolytic sensitizers.

The albuminolytic sensitizers are not carried down mechanically with the precipitates in the precipitin reactions. We see no reason for not considering them identical with the precipitins themselves, bodies which combine with the dissolved antigens and, as in other colloidal reactions, lead to precipitation when definite quantitative relations are observed.

The undiminished precipitating value of a bacterial antiserum after considerable amounts of bacterial amboceptor have been absorbed out of it, would point against the identification of agglutinins with precipitins. However, this question must be subjected to further study.

It is thus clear that only the cytolytic sensitizer can enter into combination with the washed bacterial cell, while it is probable that both cytolytic and albuminolytic sensitizer may combine with the dissolved ingredients of a bacterial filtrate. Whether or not they combine with the same constituent of such an antigen cannot be decided by our experiments.

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